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Kala-azar in Nepal: from clinical evidence to control

Suman RIJAL

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GLOSSARY OF ACRONYMS

AIDS	Acquired immune deficiency syndrome
CFR	Case fatality rate
DAT	Direct agglutination test
DDT	Dichlorodiphenyltrichloroethane
EDCD	Epidemiology and Disease Control Division
FGT	Formol-gel test
HIV	Human immunodeficiency virus
IRS	Indoor residual insecticide spraying
ITN	Insecticide impregnated nets
KAtex	Latex agglutination test
LCA	Latent class analysis
LLIN	Long-lasting-insecticide-treated-nets
MOH	Ministry of Health
NMEP	National Malaria Eradication Programme
PCR	Polymerase chain reaction
PHC	Primary health care centre
PKDL	Post kala-azar dermal leishmaniasis
Sb^v	Pentavalent antimony
SSG	Sodium stibogluconate
VL	Visceral leishmaniasis
WHO	World Health Organization

SUMMARY

Visceral leishmaniasis (VL) is a fatal vector borne disease caused by the protozoon *Leishmania* and transmitted by the bite of a sandfly. More than 50% of the annual worldwide cases occur in the poor population living in remote rural regions of the Indian subcontinent. In Nepal, a quarter of the country's population is estimated to be at risk of the disease.

With no vaccine available, the control of this disease has depended on vector control and early diagnosis with appropriate treatment of cases. With limited impact of the former the latter is presently considered the most effective strategy although there are some unresolved issues in its implementation.

Microscopy for *Leishmania donovani* bodies, considered the gold standard for diagnosis, is not feasible in the peripheral health facilities of these endemic countries and recommendations for the serological tests, direct agglutination test (DAT) and rK39 strip test, have not been forthcoming due to variations in the published estimates of test accuracy. Pentavalent antimony compounds *e.g.* sodium stibogluconate (SSG), the recommended 1st line therapy in most places including Nepal, has shown progressive decreasing efficacy in the adjoining state of Bihar, India.

With this background we conducted clinical and epidemiological research to generate evidence for more rational recommendations for the case management of VL particularly for Nepal. The work describes the burden of VL in Nepal, the application of the novel diagnostic tests for early diagnosis of VL and the efficacy of the current VL treatment in Nepal.

The research work was conducted over a period of 6 years at the B.P. Koirala Institute of Health Sciences, a university hospital, located in the Terai (lowlands),

which has established itself as a specialized VL centre attracting patient from a wide area.

The clinical and epidemiological profile of VL patients admitted from 1999 to 2004 showed that the VL epidemic in Nepal is well established with no indications suggestive of any decline. Moreover there are also signals indicating a possibility of extension of the epidemic to newer areas, which needs to be urgently examined, along with an increasing trend of cases in the urban regions. We studied the economic cost of VL to the affected households in a VL focus and showed how catastrophic and devastating an episode of VL could be to these households. A significant proportion of the cost was incurred even before starting the specific VL treatment.

The lack of access to early treatment has been related to the unavailability of a test that can be easily applied at the level of the peripheral health facility. We prospectively evaluated 3 novel tests: DAT, rK39 strip test and, a urine antigen detection agglutination test (KAtex) in patients who were clinically suspect of VL. All the tests were first studied at a tertiary level hospital and the rK39 and the KAtex were further evaluated at a peripheral level hospital. We showed that both the DAT and the rK39 had good sensitivity and specificity at the tertiary level hospital though the sensitivity of the KAtex was too low to be useful. At the peripheral level hospital the rK39 strip test showed good sensitivity, specificity and reproducibility and could be recommended as a diagnostic test.

We showed that the efficacy of SSG was too low, especially in cases coming from districts close to the high antimony resistance focus in Bihar, India. Similarly, the case fatality rate was also much higher in cases from this region in comparison to the others. We also documented an outbreak of cardiotoxicity that occurred on the use of a particular brand of generic SSG which emphasizes the need for rigorous quality control of these drugs.

The VL control program in Nepal does not appear to be able to curtail the present epidemic with its current strategy and remedial measures need to be considered. The rK39 strip test would allow for accurate diagnosis at a peripheral health facility and thus also aid in accessing early treatment. Efficacy of the present 1st line therapy, SSG, in Nepal is too low to be acceptable and warrants a revision of the present drug policy. This reduced efficacy of SSG may also indicate the spread of SSG resistant strains from neighbouring Bihar, India but this needs confirmation by drug efficacy monitoring studies. The VL epidemic in Nepal is closely linked to the Indian epidemic in Bihar so a close collaboration within the region would be essential. The recently launched "Kala-azar elimination programme in South East Asia" provides an excellent opportunity to strengthen the control programme and also help in building regional collaborations.

SAMENVATTING

Viscerale Leishmaniasis (VL) is een dodelijke infectieziekte veroorzaakt door een protozoön, *Leishmania sp.,* dat wordt overgebracht door de beet van een zandvlieg (*Phlebotomus sp*). Meer dan de helft van het aantal gevallen op wereldschaal treft de arme bevolkingsgroepen in afgelegen landelijke gebieden van het Indische subcontinent. Men schat dat in Nepal een kwart van de bevolking een potentieel risico loopt om deze ziekte te krijgen.

Aangezien er geen vaccin voorhanden is, bestaat de bestrijding van deze infectieziekte voornamelijk uit vroegtijdige diagnose en behandeling van de gevallen en vector controle.
Dat laatste heeft een zeer beperkte impact, en daarom wordt vroegtijdige opsporing momenteel als de meest efficiënte strategie beschouwd. Niettemin blijven er nog een aantal onopgeloste vragen wat betreft de uitvoering van deze strategie.

Microscopisch aantonen van *Leishmania donovani* wordt als gouden standaard voor diagnose beschouwd, maar die technieken zijn niet haalbaar in de perifere gezondheidsdiensten van de endemische landen. Officiële aanbevelingen over de toepassing van serologische tests, als directe agglutinatie tests (DAT) en rK39 immunochromatografische dipstick tests, blijven uit wegens variaties in de gepubliceerde ramingen van de test nauwkeurigheid. Pentavalente antimoon derivaten, b.v. natrium stibogluconaat (SSG), zijn de aanbevolen eerstelijntherapie in de meeste landen en dit met inbegrip van Nepal, maar deze therapie heeft in de aangrenzende staat van Bihar, India, geleidelijk zijn doeltreffendheid verloren. In deze context werd er klinisch en epidemiologisch onderzoek verricht, om als basis te dienen voor meer rationele aanbevelingen voor het VL beleid, in het bijzonder voor Nepal.

Dit werk beschrijft de zware druk die VL legt op de getroffen gemeenschappen, de evaluatie van nieuwe tests voor vroegtijdige diagnose van VL en de doeltreffendheid van de huidige VL behandeling in Nepal. Het onderzoek werd over een periode van 6 jaar uitgevoerd in het B.P. Koirala Institute of Health Sciences (BPKIHS), een universitair ziekenhuis dat in de Nepalese Terai gelegen is, de vlakte die zich uitstrekt aan de voet van de Himalaya. BPKIHS heeft zich tot een gespecialiseerd VL centrum ontwikkeld en trekt patiënten van heel de regio aan.

Het klinisch-epidemiologisch profiel van VL patiënten die van 1999 tot 2004 werden opgenomen in BPKIHS, toont aan dat de VL epidemie zich reeds sterk heeft doorgezet in Nepal, zonder enige aanwijzing van een eventuele verbetering. Bovendien zijn er ook signalen die wijzen op een mogelijke uitbreiding van de epidemie naar nieuwe gebieden. Deze eventuele uitbreiding dient dringend te worden onderzocht, samen met de stijgende tendens van VL gevallen in stedelijke gebieden.

We bestudeerden de financiële gevolgen van de ziekte bij getroffen gezinnen in een VL transmissiefocus en toonden aan welke verreikende gevolgen VL kan hebben bij deze gezinnen. Het gezin spendeert reeds een belangrijk deel van de ziekte-uitgaven vóór de aanvang van de eigenlijke VL behandeling.

Het gebrek aan toegang tot vroegtijdige behandeling is een gevolg van het ontbreken van een eenvoudige diagnostische test die op het niveau van de perifere gezondheidsdiensten kan worden ingezet. Wij hebben 3 nieuwe tests geëvalueerd: DAT, rK39 en een urine antigeen detectie test (KAtex) bij mensen die klinisch verdacht waren van VL. De drie tests werden eerst geëvalueerd in het tertaire ziekenhuis, de rK39 en KAtex werden verder geëvalueerd in een perifeer districtsziekenhuis. We toonden aan dat zowel DAT als rK39 een goede gevoeligheid en specificiteit hebben in het universitair ziekenhuis, terwijl de gevoeligheid van KAtex als te laag werd beoordeeld. In het perifere districtsziekenhuis toonde de rK39

test goede gevoeligheid, specificiteit en reproduceerbaarheid en zou derhalve als diagnose test kunnen worden aanbevolen.

Verder toonden we aan dat de doeltreffendheid van SSG ontoereikend was, vooral in gevallen die uit het grensgebied met Bihar, India, komen, dat een hoge graad van SSG resistentie rapporteert. Bovendien was de letaliteit van de ziekte bij deze mensen ook veel hoger in vergelijking met die in de andere gebieden. Tijdens de onderzoeksperiode werden we geconfronteerd met een probleem van iatrogene mortaliteit omwille van de cardiotoxiciteit van een lot van generische SSG van een bepaalde producent. In onze rapportering hierover hebben we de noodzaak tot strenge kwaliteitscontrole van deze generische medicatie benadrukt.

Het VL- bestrijdings programma in Nepal krijgt de huidige epidemie duidelijk niet onder controle, en daarom moeten er nieuwe maatregelen worden genomen. Het invoeren van de rK39 test op de eerste lijn van de gezondheidsdiensten zou een grote verbetering zijn, omdat het de toegang tot vroegtijdige behandeling zou verbreden. Volgens onze gegevens is de doeltreffendheid van de huidige eerstelijntherapie, SSG, in Nepal te laag om nog aanvaardbaar te zijn en dit vraagt om een herziening van het huidige geneesmiddelenbeleid. De verminderde doeltreffendheid van SSG kan wijzen op de verspreiding van SSG resistente *L.donovani* stammen vanuit het naburige Bihar, India , maar dit moet via klinische en parasitologische research worden bevestigd. De VL epidemie in Nepal is nauw verbonden met de Indische epidemie in Bihar, zodat een nauwe samenwerking tussen de gezondheidsautoriteiten van deze landen zich opdringt. Het onlangs door de Wereldgezondheidsorganisatie gelanceerde "Kala-Azar eliminatieprogramma in Zuidoost-Azië" opent perspectieven om die regionale samenwerking te versterken.

CHAPTER 1:

OVERVIEW OF VISCERAL LEISHMANIASIS OR KALA-AZAR

1.1 Visceral leishmaniasis, the disease

Etiology and pathogenesis

Leishmaniasis is a vector-borne parasitic disease, with a spectrum of clinical manifestations: visceral, cutaneous and mucocutaneous. The clinical form of the disease depends not only on the species of *Leishmania*, but also on the complex interactions resulting from the parasite's invasiveness, tropism and pathogenicity and the host's genetically determined cell-mediated immune response. Visceral leishmaniasis (VL) or kala-azar is caused by *Leishmania donovani* in the Indian subcontinent and east Africa, by *L. infantum* in the Mediterranean region and west-Africa and *L. chagasi* in Latin-America (Lainson & Shaw JJ 1987). According to some authors the latter species is considered identical to *L. infantum*. These pathogens are transmitted exclusively by the bite of a female phlebotomine sandfly. For *L. donovani*, man is the only reservoir, whereas for *L. infantum* and *L. chagasi*, the domestic dog is the main reservoir. The leishmanial parasite exists in two morphological forms: the promastigote form (with flagella) extracellularly in the sandfly and culture medium and the amastigote form (oval, non-motile cell), intracellularly in the vertebrate host.

The promastigotes, after the inoculation in the skin (dermis) by the sandfly, are phagocytosed by the macrophages and get converted to amastigotes that multiply inside the acidic parasitophorous vacuoles. They then disseminate through the lymphatic and vascular system and infect macrophages throughout the reticuloendothelial system. *Leishmania* use the macrophage like a 'Trojan horse' (Jeronimo et al. 2005). Increase in the number of infected macrophages in the organs (mainly liver and spleen) leads to their hypertrophy.

The clinical outcome after a leishmanial infection is dependent on the type of immune response. Symptomatic VL can be viewed simplistically as a failure of the specific cell-mediated immunity leading to lack of activation of the macrophages to overcome the infection. These patients have anergy to leishmanial antigens as

indicated by a negative delayed hypersensitivity skin test while high titres of antileishmanial antibodies are produced that are not protective (Neva 1990). With recovery from VL, protective immunity develops and the majority of patients display a positive delayed hypersensitivity skin test.

Immunosuppression including HIV infection and suppressive therapy for organ transplantation are associated with increased risk of VL (Fernandez et al. 1987). Malnutrition which lowers cell mediated immunity has also been shown to be a risk factor (Cerf et al. 1987).

Transmission cycle of leishmania

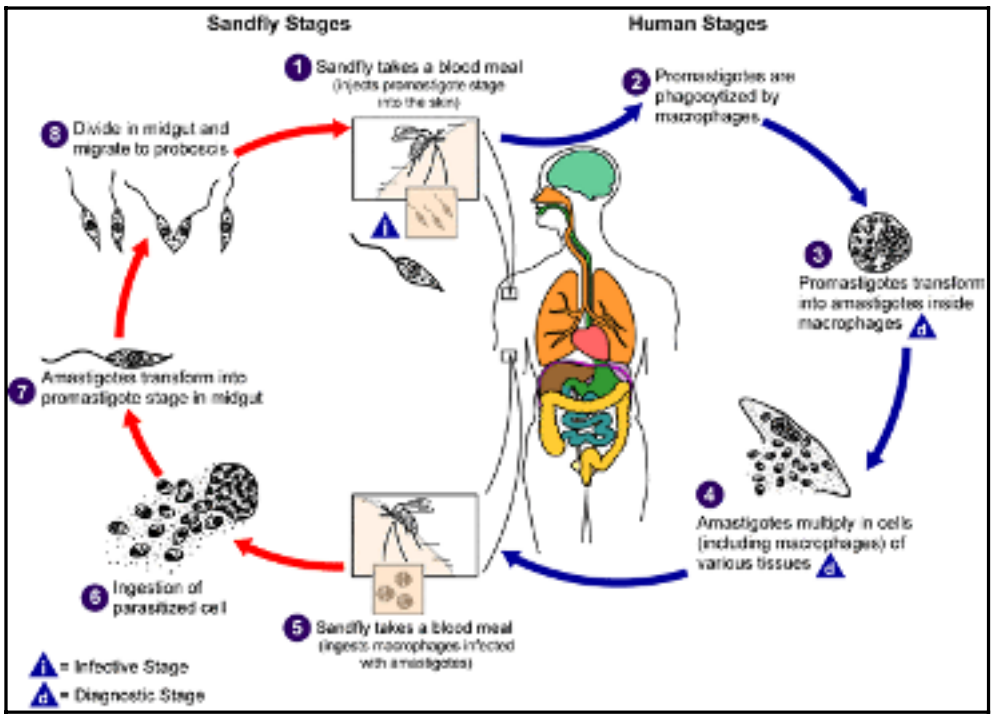
Depending on the transmission characteristics, two types of VL can be distinguished: a zoonotic and an anthroponotic form. In the former, there is an animal reservoir (dogs, foxes, rodents) where the parasite is maintained and man being an occasional host, and in the latter the parasite is exclusively maintained in a man-vector-man cycle in the absence of any animal reservoir (Figure 1).

In the Indian subcontinent, VL is of the anthroponotic type and distribution, experimental infection and transmission by bite have proven *Phlebotomus argentipes,* a peridomestic sandfly, to be the vector (Lainson & Shaw JJ 1987). Cracks in the mud huts plastered with cow dung in the rural areas of the subcontinent provide a good resting place for the sandflies. Density of the sandflies is dependent on climatic and environmental conditions. In Nepal, vector density was observed to start rising from April, peaking in May and then a decline from September to a negligible level in December (Das 1998). Biting is usually nocturnal with the highest activity between 21.00 to 01.00 hours (Dinesh et al. 2001).

The disease is spread when female sandflies ingest amastigotes while taking a blood meal from an infected mammal. These transform to promastigotes within the insects' gut, migrate to the proboscis and are deposited in the dermis of the new host when the insect next engorges. The mammalian host is infectious to the sandfly as long as the parasite persists in the circulating blood or in the macrophages within the

dermis. In anthroponotic VL, infectivity to the sandfly may persist even after clinical recovery from VL, especially when the patient develops post kala-azar visceral leishmaniasis (PKDL).

Fig.1: *Leishmania* life cycle



(Source: CDC's website, laboratory identification of parasite)

Person to person transmission has been reported through transfusion of contaminated blood (Grogl et al. 1993; Shulman 1994), accidental needle stick injuries (Evans & Pearson 1988; Herwaldt & Juranek 1993) and sharing of contaminated needle syringes in intravenous drug users (Cruz et al. 2002). Congenital *Leishmania* transmission has also been rarely seen (Eltoum et al. 1992; Meinecke et al. 1999).

Clinical features

Not every infected individual progresses to VL disease, there is a spectrum from asymptomatic infection over subclinical disease to the full blown VL syndrome

(Pearson & Sousa 1996). Asymptomatic and subclinical forms are frequent as shown during epidemiological surveys in various foci *e.g.* Italy (Pampiglione et al. 1975), Brazil (Badaro et al. 1986a; Carvalho et al. 1992; Evans et al. 1992), Kenya (Schaefer et al. 1995), Ethiopia (Ali & Ashford 1994) and Sudan (Zijlstra et al. 1994; Khalil et al. 2002). The ratio of asymptomatic to clinical cases has varied from 1:2.4 to 18:1. Subclinical or mild forms were also described in the veterans of Operation Desert Storm (Magill et al. 1993). Leishmanial parasites have been cultured from healthy blood donors living in a VL endemic area in southern France (Le Fichoux et al. 1999). The exact role of these asymptomatic and subclinical cases in the transmission of VL is not clear. Some of the subclinical infections may subsequently progress to clinical disease when the immune status changes.

Clinical manifestations of VL or kala-azar are similar throughout the world. The incubation period, though difficult to evaluate precisely, is generally 2 to 6 months but can range from 10 days to up to 10 years (Dedet JP & Pratlong F 2003). Clinical disease may first become symptomatic years after exposure in those who become immunocompromised (Badaro et al. 1986b).

Classical VL usually has a sub-acute or chronic course presenting with an insidious onset of fever, weakness, loss of appetite, weight loss, abdominal distension due to hepatosplenomegaly and leukopenia. Fever, the commonest symptom, may be remittent, intermittent with twice-daily spikes or less commonly continuous. Lymphadenopathy is common in Sudan but not in the Indian subcontinent. Hyper pigmentation is seen in some patients in the Indian subcontinent – which led to the name kala-azar, meaning black fever in Hindi - and this may be related to increase in melanocytes (Dedet JP & Pratlong F 2003). A more acute course of the disease is described in immunologically naïve populations (Hashim et al. 1994).The symptoms usually persist for weeks to months before the person seeks medical attention. In advanced disease secondary bacterial infections are common (pneumonia, tuberculosis, septicaemia, dysentery or measles) which can be fatal. Other causes of death in VL may be due to haemorrhage as a result of thrombocytopenia. The differential diagnosis of classical kala-azar includes malaria, relapsing fever, typhoid

fever, tuberculosis, AIDS, brucellosis, cirrhosis, lymphomas and leukaemia's (WHO 1996).

VL/HIV co-infection has been reported from 35 countries but most cases have been recorded from southwestern Europe. There is evidence of increasing cases from east Africa and the Indian subcontinent (Desjeux & Alvar 2003; Hailu & Berhe 2002).VL is observed as an opportunistic infection in 3 -7% of persons with HIV infection in the Mediterranean basin. Clinical manifestations of VL are usually similar to those in HIV negative cases but may develop unusual multi-organ pathology following the dissemination of the parasites. Almost all co-infected cases are prone to relapse after VL treatment (Russo et al. 2003).

Post kala-azar dermal leishmaniaisis (PKDL), considered a complication of VL, is more commonly seen in inadequately treated VL cases (Zijlstra et al. 2000). In Sudan, it occurs in over 50% of VL cases and the lesions appear shortly after the symptoms of VL subside (0 to 6 months) (Zijlstra et al. 2003). In the Indian subcontinent it is seen in 5-10% of VL cases usually after an interval of 1 to 2 years after treatment, though up to 20 years have been reported in exceptional cases (Ramesh & Mukherjee 1995). The skin lesions are characterized by a spectrum of manifestations ranging from depigmented macules to wart-like nodules over the trunk and face, mimicking the lesions of leprosy. In Sudan, most cases cure spontaneously but chronic and severe cases require at least 4 months of the standard antimonial regimen. PKDL lesions being full up with parasites infectious to the sandflies, are considered to play an important role in disease transmission between outbreaks (Addy & Nandy 1992; Dye 1992; Dye & Wolpert 1988).

1.2 Epidemiology and public health importance

Historical perspective of VL in the Indian subcontinent

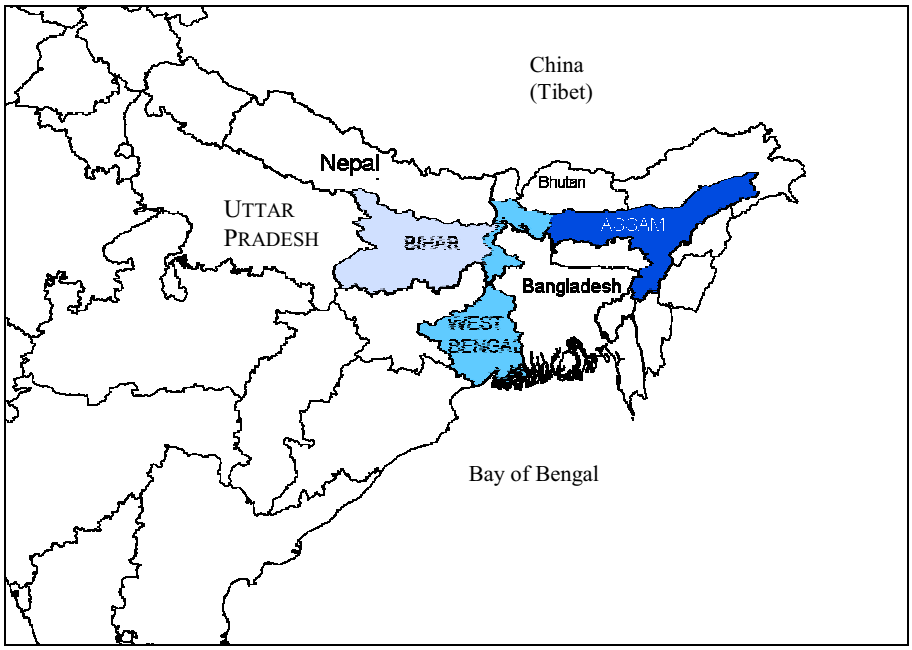
There is no mention of any disease akin to kala-azar in ancient Indian medical literature. The earliest reference to this disease was made in the annual report of the

inspector general of civil servants for 1872, in which he has quoted Dr French, the civil surgeon of Burdwan district in the state of Bengal (Sanyal RK 1985).

"*Dr French is submitting his report, enters into the history of the epidemic from its appearance in Mahmadpur in the Jessore district* **(presently in Bangladesh)** *in 1824 when the disease first attracted attention tracing it into the Nuddea district in 1832 or 1833 , then to the Hooghly district in 1857 and subsequently in 1862 he considers that the epidemic reached Burdwan district.....The type of disease described is that ordinarily known as 'malarious intermittent fever' not infrequently the intermittent passes into the remittent, and the latter into the continuous type, and either may be followed by dysentery, enlargement of spleen or dangerous local derangement and disorganisation*".

This was apparently the description of the first epidemic in the Indian subcontinent. The fever was locally referred to as "Jwar-Vikar' (febrile disorder) and "Burdwan fever".

Figure 2: North East India along with Nepal and Bangladesh



The epidemic swept through western and central Bengal in eastern India (figure 2) leaving a trail of devastation and turning some areas of the Hooghly and Burdwan valleys (in Bengal) into 'a valley of the shadow of death' with a death toll of 75,000 lives in 3 years (Sen Gupta PC 1944). After establishing itself in the plains of Bengal, kala-azar spread along the lines of the two major rivers, Brahmaputra and the Ganges and its tributaries, which were the main lines of communication, to Assam in the north-east and Bihar in the west. A full scale epidemic ravaged the valleys of Assam from 1890 to 1900 with mortality rates up to 95% (Peters 1981). At a time when the population growth was 15-20 % per decade, the epidemic in Nowgong district in Assam caused a decrease of over 30% of the population during this epidemic. In Purnea district, Bihar, a disease locally called "kala-dukh" meaning the "black misery" existed towards the end of the nineteenth century which was identified as kala-azar by Ross in 1899 (Sanyal RK 1985).

Only in 1903 , Leishman and Donovan separately described the presence of a parasite in cases of kala-azar, which was named *Leishmania donovani (LD)* by Laveran and Mesnil the same year (Wenyon CM 1926).Diagnosis of kala-azar was thus established by the demonstration of these LD bodies. The formol-gel or aldehyde test was accidentally discovered by Napier in 1921 (Napier LE 1922). He observed that the addition of a few drops of formalin to the serum of kala-azar patients resulted in "egg white" solidification. This formol-gel test continues to be used as a diagnostic test for kala-azar, in many of the peripheral health centres in the Indian subcontinent.

The next kala-azar epidemic, from 1917 to 1929, raged through the eastern part of the entire subcontinent and with an intensified situation in Bihar from 1935 to 1937 (Peters & Prasad LSN 1983). It was during this epidemic, that for the first time in the subcontinent, antimony was used on a large scale in the form of tartar emetic which was soon replaced by urea stibamine the first pentavalent antimony (Sb^v) compound introduced by Brahmachari in 1922. This was a remarkable advance in the fight against kala-azar, which reduced the mortality from 90 to 95% to less than 5% in treated cases. The heavy toll of the disease including the economic impact on the tea

plantations in the state of Assam led to the constitution of the "the kala-azar commission" which submitted historic reports in 1926 and 1932 outlining the dynamics of transmission of the parasite through sandfly vectors (Sanyal RK 1985).

The number of kala-azar cases started to decline from mid 1950's to a point of almost disappearance within a decade. The causes of disappearance have been critically reviewed and identified as: 1. effective treatment of all cases; 2. increase in herd immunity and consequent inter-epidemic lull; and 3. impact of dichlorodiphenyltrichloroethane (DDT) spraying in the National Malaria Eradication Programme (NMEP) (Sanyal RK et al. 1979b).

A decade after stoppage of the DDT spraying the disease again gained epidemic proportions in north Bihar reaching a maximum in 1977 with an estimated 100,000 cases (Thakur 1984). This resurgence has been linked to a continuing low level of transmission along with the persistence of many cases of PKDL. The PKDL cases, most of whom were not recognized or not treated, were a potential source of infection, and acted as a reservoir (Sanyal RK et al. 1979b; Singh 1968). Since then, kala-azar has established itself as an endemic disease in north Bihar and adjoining parts of West Bengal in India, with outbreaks like the one in 1991-1992 (Bora 1999).

Situation in Nepal

Nepal is ecologically divided from north to south into three regions: mountain, hill and the terai (lowlands). The terai has a subtropical climate and is known to harbour most tropical diseases.

There has been no documentation of the kala-azar epidemics of the nineteenth century from Nepal. Till 1953 the country was closed to the outside world. Napier in 1926 reports of a large proportion of VL cases from Calcutta to be Nepalese patients (Napier LE 1926). However, it is unclear if these were Nepalese residing in India or Nepalese travelling to India for treatment, which was a common practice. The first documented evidence of kala-azar in Nepal was made only in 1953 by an Indian scientist, NGS Raghavan, who after a survey for vector borne diseases in 1949

claimed that kala-azar was endemic in the entire terai (Shrestha & Pant 1994).There is also no documentary evidence to show the magnitude of the problem before the start of the DDT spraying in the NMEP in 1958. Even there was no mention of kala-azar in the first report of "Health and health administration in Nepal" published by the Directorate of Health Services in 1969 (Shrestha & Pant 1994). The first officially recorded case of VL in Nepal was in 1980 from Dhanusha district (Bista 1998). Since then there has been a steady increase in the reported cases and presently 12 districts are identified to be endemic with more than 5.5 million people estimated to be at risk of the disease (EDCD 2002b). From 1980 to 1989 the incidence rate per 100,000 person-years remained below 10. Since then it has been increasing steadily and in the last few years it has been reported from 43 to 55 per 100,000 person-years. The case fatality rate (CFR) for VL has been reported to be around 1% in the last few years. However, these surveillance data from Ministry of Health (MOH) are considered to be underestimated as it does not include cases treated outside the public health facilities (Bista 1998). Little epidemiological research has been conducted from Nepal. One small cross- sectional survey in an endemic focus in eastern Nepal showed *Leishmania* infection rate of 7.5% documented by a serological test (Schenkel et al. 2004) and a qualitative survey conducted in 2 villages in the endemic region revealed poor knowledge about the transmission and symptoms of kala-azar (Koirala et al. 1998).

Control program in Nepal

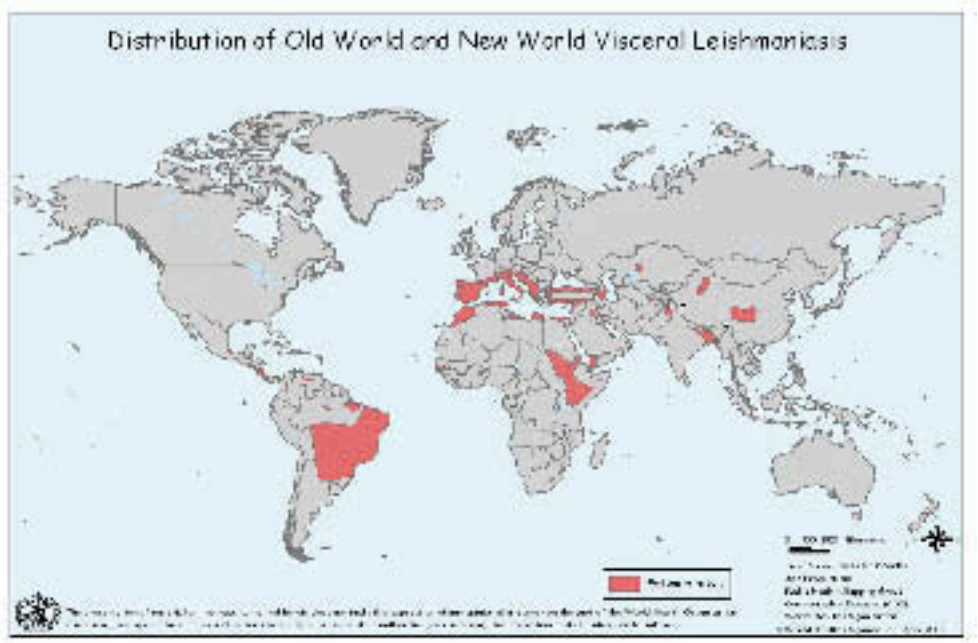
A kala-azar control programme under the Epidemiology and Disease Control Division (EDCD), MOH is ongoing since 1993. The goal of the programme is to reduce kala-azar to less than 1 in 100,000 person-years and PKDL cases to zero by the year 2018 (EDCD 2002a).The strategy for control is based on early diagnosis and treatment of VL along with indoor residual insecticide spraying (IRS). Case detection is done passively in those who present to the public health facilities. The recommended diagnostic test is bone marrow microscopy at the level of the district

hospitals and above and rK39 striptest (recommended from 2002) at the level of primary health care centre (PHC). Sodium stibogluconate (SSG) is the recommended 1st line therapy that is prescribed at the level of PHC or higher health facility but can be continued at the health posts. The 2nd line therapy, amphotericin B, is available at the level of district hospitals or higher. In practice as parasitological diagnosis is not feasible in the majority of the district hospitals along with the lack of regular supply of the rK39 striptest, treatment for VL is started on the basis of the clinical features and the formol-gel test (FGT), a non specific test for VL. As a regular system of monitoring treatment is lacking, the outcome of VL therapy is not well documented.

Frequency and burden of the disease

The leishmaniasis constitutes one of the 10 entities of the World Health Organization /Tropical Disease Research list of neglected infectious diseases that disproportionately affects poor and marginalised populations. Visceral leishmaniasis (VL) has been reported from over 60 countries but more than 90% of the estimated 500,000 annual new cases occur in India, Bangladesh, Nepal, Sudan and Brazil (TDR 2005). The Indian subcontinent accounts for 60% of the global burden (in terms of disability adjusted life years lost) caused by VL (Murray & Lopez 1996).
Moreover, as VL has a very focal distribution and preferentially affects the poor living in remote areas with difficult access to health care (Guerin et al. 2002), the aggregated figures do not reflect the real burden of disease in these affected communities. Reported incidence rates of kala-azar in endemic areas vary between 2/1000 person-years in Kenya (Schaefer et al. 1995), 14/1000 person-years in Ethiopia (Ali & Ashford 1994) to 40/1000 person-years in a community survey in eastern Sudan (Zijlstra et al. 1994; Khalil et al. 2002). In Bangladesh, an incidence of 20/1000 persons-years was recorded in a recently conducted community survey (Ahluwalia et al. 2003). The global annual VL mortality is officially estimated at 59,000, however this is considered severely underestimated (Desjeux 2004).

Figure 3: Worldwide distribution of VL



VL is a fatal disease and the devastating effects seen in the Indian epidemics before the availability of the Sb^v compounds is quite illustrative. Even more recently, Seaman et al. (1996) described a severe epidemic in Sudan where over the period 1884 to 1994, an estimated 100,000 VL deaths occurred in a group of 280,000 people living in Western Upper Nile province affected by civil war and displacement of populations. A recent community survey from Bangladesh reported a case fatality rate of 19% among adult females with kala-azar (Ahluwalia et al. 2003).

With most of the VL in the Indian subcontinent affecting the economically active population (15 to 45 years), in every member of a family where a VL case occurs, many days of productive life are lost due to this severely debilitating disease.

1.3 Control strategies

The resurgence of leishmaniasis world-wide has been attributable to the appearance of new risk factors in addition to an increase in the previously identified ones

(Desjeux 2001). The major risk factors for VL include: 1. low socio-economic level and cultural practices; 2. environmental factors e.g. moist soil, organic debris near household etc.; 3. changing ecology *e.g.* new settlements especially in urban areas, civil war; 4. migration of population *e.g.* cross border migration between India and Nepal; 5. emergence of VL/HIV co-infections, and 5. ineffectiveness of control programmes. At the community level, the proximity to a previous kala-azar patient was an increased risk (Bern et al. 2005).

The intervention strategies for prevention or control depend on the specific eco-epidemiological situation with control strategies tailored to the two main epidemiological entities: anthroponotic and zoonotic.

Historical examples of effective VL control are available though limited. VL was a major parasitic disease in north-east China with 530,000 cases reported in 1951 (Guan & Shen 1991). From 1950-1958 a strenuous control campaign including treatment of cases and spraying of insecticides was launched. The disease was largely brought under control in the plains region where anthroponotic form had reigned but was not quite successful in the mountainous and hilly regions having zoonotic VL.

In one district in Bihar, India there was a 65% decrease in the number of VL cases 2 years after an intensive integrated control campaign (Saxena et al. 1996). The intervention consisted of active surveillance for case detection and treatment combined with residual insecticidal spraying in the households along with information, education and communication activities.

The several theoretically possible control interventions include: 1. vector control and personal protection; 2. reservoir control; 3. immunoprophylaxis; and 4. early diagnosis and treatment. An overview of each is given below.

Vector control and personal protection

As detection of the sandfly breeding sites is generally difficult, control measures against immature forms are not feasible (Alexander & Maroli 2003). Indoor residual

insecticide spraying (IRS) of households is the most widely used intervention for controlling sandflies that are endophilic (rest mostly indoors after feeding), and have yielded good results in India (Joshi & Rai 1994; Kaul et al. 1994). However cost of the insecticides, suitable public health infrastructure (spraying equipment trained personnel), the low acceptance by the community (Gupta PC 1975) compromise the longer-term effectiveness and sustainability of this intervention.The extensive DDT spraying during the NMEP in the 1960's in the Indian subcontinent along with malaria also decreased the cases of VL to negligible levels but the cessation of spraying was followed by major epidemics of VL.

Insecticide impregnated nets (ITN) were shown over the past two decades as one of the most effective methods of reducing man-vector contact in intra- and peri-domiciliary transmission of vector-borne diseases. ITN is considered an effective, cheap and sustainable method in the control of malaria.

A case control study, investigating factors associated with VL, found a 70% less probability of getting VL in those who used untreated nets compared to people who did not use any nets. (Bern et al. 2000). Treatment of the wide mesh nets with insecticides enhances the effect and has shown to decrease sandfly bites by 64 to 100% (Davies et al. 2003). Field studies in Syria, Iran and Afghanistan have shown the ITN to be effective in cutaneous leishmaniasis, but no such studies have been undertaken for VL so far.

Control of reservoir

Control of leishmaniasis by controlling the reservoir hosts has been recommended for zoonotic cutaneous as well as visceral leishmaniasis (WHO 1990). In zoonotic VL annual surveys for canine infection along with culling of the dogs has been a part of the control programme in Brazil. But as this has failed to reduce the number of notified cases it has let to scepticism of the effectiveness of this programme (Ashford et al. 1998). Other strategies for reservoir control have been the application of topical insecticides (dipping the dog in insecticide or application of insecticidal lotions)

(Reithinger et al. 2001) which can substantially reduce the number of sandfly bites in the dogs. A novel experimental approach using pyrethenoids treated collars not only reduced the risk of infection to the dogs but also to the children living in the same village (Gavgani et al. 2002). In anthroponotic VL, where humans are the only reservoir, active case detection and treatment of VL and PKDL is recommended.

Vaccines

At present there is no vaccine available for any type of leishmaniasis. Leishmaniasis is considered a unique parasitic disease as a single vaccine would have the potential to protect against more than one species and be successful for both treating and preventing disease. Pioneering work on 1st generation leishmanial vaccines, using whole organism preparations, demonstrated limited potential against both cutaneous and visceral leishmaniasis (Khalil et al. 2000; Modabber 1995). Over the past 2 decades several antigens have been identified and characterized that could be potential vaccine candidates. Animal studies have demonstrated effective prophylactic and therapeutic protection against multiple forms of leishmaniasis. The 1st defined vaccine for leishmaniasis in human clinical trials, Leish 111f , has completed phase 1 safety testing in normal human subjects and is currently undergoing therapeutic trials in cutaneous and mucocutaneous leishmaniasis in Latin America (Coler & Reed 2005). However, for VL the situation is less promising. Human trials of vaccines against VL are likely to follow only from successful outcomes of those against cutaneous leishmaniasis (Davies et al. 2003).

Early diagnosis and control

In the background of limited efficacy of vector control and in the absence of a vaccine, early case detection and treatment is considered the basic intervention to limit morbidity and mortality in anthroponotic VL. Active surveillance, though difficult to organise and implement, was observed to be more efficient compared to

insecticide spraying during the epidemic of 1976-1978 in Bihar state, India (Sanyal RK et al. 1979a). Moreover, special attention should also be given to detection and treatment of PKDL cases, an important source for transmission but has remained a neglected part of the control programme (Thakur & Kumar 1992).

The diagnosis and treatment of VL is dealt with in the following section.

1.4 Management of visceral leishmaniasis

Diagnosis

Microscopic demonstration of the amastigotes of *L.donovani* from tissue aspirates continues to be the recommended method of diagnosis ever since the discovery of the parasite by Leishman and Donovan over a century ago. Executed by a trained microscopist this technique is 100% specific but the sensitivity varies with the type of aspirate : spleen tissue being the most sensitive, over 95% (Bryceson 1996; Thakur 1997; Zijlstra et al. 1992). In addition to being an invasive and complex procedure (WHO 1996), a small risk of fatal haemorrhage remains (Chulay & Bryceson 1983). Lymph node and bone marrow aspirations are safer procedures but their sensitivity has been shown to be only 58 to 64 % and 70 to 86 % respectively (WHO 1984; Zijlstra et al. 1992). A major limitation in establishing parasitological diagnosis in VL endemic countries is the lack of expertise (physician to perform the procedures and the laboratory technician to stain and read the slides) outside reference tertiary hospitals or specialized treatment centres.

In the pursuit for alternatives to parasitology, several serological tests have been developed and evaluated in the field over the last 2 decades. Their main advantage is non-invasiveness but they do not discriminate between clinical, subclinical or past infection and also cross-reaction with other pathogens is possible (Hommel et al. 1997). The Direct Agglutination Test (DAT) developed by El Harith et al. (1986; 1988) proved to be very accurate under laboratory conditions (el Safi & Evans 1989; Joshi et al. 1999; Singla et al. 1993). In field conditions, low specificity values were reported in series of clinical suspect patients with a range depending on the

reference test used, between 58 % (Boelaert et al. 1999a) and 72 % (Zijlstra et al. 1991). The effectiveness of DAT proved satisfactory in a large multi-centre study (Boelaert et al. 1999b). Another problem of DAT has been its low reproducibility (Boelaert et al. 1999c) which is due to the fragility of the antigens. The antigens which are produced in a few laboratories in Europe have a risk of losing potency, due to heat and shaking, during transport to the endemic countries. This problem has been overcome by the development of the freeze dried version (Meredith et al. 1995; Zijlstra et al. 1997).

More recently a test based on 39- amino acid repeat recombinant leishmanial antigen from *Leishmania chagasi* (rK39) has been introduced in an ELISA (Badaro et al. 1996; Zijlstra et al. 1998), and later in a lateral flow dipstick format (Sundar et al. 1998a). The latter is very easy to use in the field and an initial study showed 100% sensitivity and 98% specificity. However, the performance of this strip test in subsequent studies was not consistent, showing low sensitivity (Jelinek et al. 1999; Zijlstra et al. 2001) and specificity (Veeken et al. 2003).

Recently, a urinary leishmanial antigen, a low molecular weight heat stable carbohydrate, detected in the urine of visceral leishmaniasis patients has been described (Sarkari et al. 2002). A latex agglutination test for the detection of this urinary antigen (KAtex) evaluated in laboratory trials, showed a specificity of 100% and sensitivity between 64 to 100% (Attar et al. 2001). An antigen detection test could be promising for both diagnosis and prognosis.

The application of molecular biology techniques like polymerase chain reaction (PCR) for diagnosis of VL have been validated using tissue aspirates as well as blood samples (Adhya et al. 1995; Andresen et al. 1997; Osman et al. 1997). The sensitivity has been shown to be very high, being able to detect the equivalent of even less than one parasite per sample (Salotra et al. 2001). It has been shown to be successful in the diagnosis of immuno-competent VL children in Italy (Cascio et al. 2002) and for monitoring of relapses in HIV co-infected patients (Pizzuto et al.

2001).The major limitation of the conventional PCR for field use is the sophisticated technique and the high cost.

In the VL endemic countries, confirmation of clinical diagnosis remains a problem. In addition, the patients delay in seeking medical attention. In a study from India, the mean time from onset of symptoms to definitive diagnosis was 7.7 months (Sundar S et al. 1991).

Treatment

Visceral leishmaniasis, a fatal disease, has a limited number of drug options available and almost all of them are far from satisfactory. This disease has recently gained attention as one of the "most neglected diseases" along with Chagas' disease and sleeping sickness due to the lack of effective, affordable or easy to use drug treatments (Trouiller et al. 2002; Yamey & Torreele 2002).

The drugs currently available for the treatment of VL include the pentavalent antimonials, sodium stibogluconate (SSG) (Pentostam®, GlaxoSmithKline) and meglumine antimoniate (Glucantime®, Aventis), pentamidine, amphotericin B and its lipid formulation AmBisome® (Gilead), miltefosine (Impavido®, Zentaris) and paromomycin.

Pentavalent antimonials (Sb^v) in use since the 1940's and despite its prolonged parenteral therapy remains the 1st line therapy in most regions due to the time tested efficacy and affordability (Berman 1997; Murray 2001).Recent evaluations comparing the branded Sb^v with the cheaper generic SSG in Sudan showed the latter to be equally effective but costing only one-fourteenth of the generic brand (Moore et al. 2001; Murray 2004; Veeken et al. 2000).The major problems with SSG have been its increasing failure rate and toxicity. The decrease in cure rates in the state of Bihar, India was first noted in the early 1980's and this was overcome by increasing the dose and duration of therapy of SSG (WHO 1984; 1990). However in the early 1990's, in spite of the use of the maximum recommended and tolerated doses (20mg/kg/day for 30 to 40 days) there was a steady decline in the cure rates to reach as low as 35%, thus its use has been abandoned in this region (Jha TK et

al. 1992; Sundar et al. 2000). In Nepal, little information is available except for one study conducted in 1996 which showed a cure rate of 93% in a cohort of 27 VL patients treated with SSG 20mg/kg/day for 30 days (Karki et al. 1998)

With the maximum tolerated doses the common toxicities include chemical pancreatitis, symptomatic pancreatitis and musculoskeletal pain in one-third to two-third of cases (Gasser, Jr. et al. 1994). Though significant cardiotoxicity is not common in patients receiving single courses (Berman 1997; Herwaldt & Berman 1992; Murray 2001), serious cardiotoxicity including deaths was observed with bad batches of some generic preparations (Sundar et al. 1998b).

The increase in treatment failure of SSG, has been linked to the use of inadequate doses (Sundar et al. 1994) and emergence of resistance strains of *L.donovani* (Lira et al. 1999). Inadequate and incomplete treatment being the major factor for the development of the latter (Bryceson et al. 1985).

Pentamidine, a diamidine, was used as a 2nd line drug especially in SSG refractory cases. However, its usefulness has been limited due to decline in its efficacy and serious toxicity (both hypo- and hyperglycaemia) (Jha et al. 1991; Thakur et al. 1991).

Amphotericin B desoxycholate, a polyene antibiotic, has proved to be highly effective with over 95% cure (Thakur et al. 1999) for the treatment of antimony resistant VL and is the recommended 2nd line therapy except in Bihar where it has become the 1st line regimen. However, its major drawbacks includes prolonged hospitalization as it needs to be given by infusion lasting at least 4 hours, toxic adverse effects requiring close laboratory monitoring and comparatively higher overall cost of treatment (Berman 1997; Murray 2001). Among the lipid formulations of amphotericin B (AmBisome®, unilamellar liposomes; Abelcet, lipid complex; Amphocil, colloidal dispersion) liposomal amphotericin or AmBisome® is by far the best available drug with respect to the efficacy and side effects (Berman et al. 1998). This has also significantly decreased the use of pentavalent antimonials in Europe (Murray 2004). Cure rates similar to amphotericin B was obtained at low dosages (2mg/kg/day for 5

days) in India (Sundar et al. 2004). However, even with these doses the cost of the drug is way beyond the reach of most VL patients.

Miltefosine, an alkylphosphocholine, originally developed as an anti cancer drug, is the first and presently the only oral drug available for VL. Clinical trials in India have shown a high cure rate (Jha et al. 1999; Sundar et al. 2002) and it remains to be seen if it has similar efficacy in other VL regions *e.g.* east Africa and South America. Gastrointestinal side effects though common (60%) are mild and transient. However, the major drawback is that it must be used with great caution in women of child bearing age as it is teratogenic in animals (Croft & Coombs 2003).

Paromomycin, an aminoglycoside antibiotic, in early clinical trials conducted in India has shown high level efficacy and low rates of adverse events (Thakur et al. 2000). If these are confirmed in further trials paromomycin has the potential to replace amphotericin B in Bihar, India and Sb^v in the other regions as a 1^{st} line therapy.

A major consideration of the choice of the drug in the treatment of VL other than efficacy and adverse events has been its cost, which is quite varied. The estimated cost of the different drugs for treating a 25 kg patient in India is: generic SSG = US$ 22; amphotericin B= US$ 60; AmBisome® (2mg/kg/day for 5 days)= US$ 800; miltefosine = US$ 100 (Murray 2001; Murray 2004). Additional cost would include the cost of hospitalisation, monitoring of therapy and the re-treatment of failures and would vary in the different regions.

Immuno-chemotherapy and new chemotherapy have been experimented (*e.g.* interferon γ, and granulocyte –macrophage colony stimulating factor), but it would be a difficult and long process for these to reach the stage of clinical application for this neglected disease (Murray 2004).

Recently, a consensus emerged that drug resistance in VL should be avoided by combination therapy combining the currently available drugs (Guerin et al. 2002). This will not only overcome the problems related to drug resistance and treatment failure but may also mitigate the toxicity with shorter regimens.

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CHAPTER 2

RATIONALE, OBJECTIVES AND PRESENTATION OF THE PUBLISHED WORK

2.1 Rationale of the research:

Lack of adequate epidemiological information has been implicated as a major hindrance for VL control efforts (Bista 1998). In Nepal in particular, little is known on the disease trend and even less on the true extent of this purported epidemic nor on the economic impact of this chronic fatal disease on the affected communities that come from the lowest socio- economic strata of the society. This information was urgently needed.

Besides, the options for control, especially for the anthroponotic form of VL *i.e.* that without animal reservoir, are limited. With no vaccine foreseen in the near future, the present strategies include vector control and early case detection with appropriate treatment. As the former has so far shown limited impact there is a wide consensus that the latter is the single most important strategy presently available (Boelaert et al. 2000; Guerin et al. 2002). However, there remain several unresolved issues for appropriate implementation of the early detection and treatment strategy.

To build an effective and efficient test and treatment strategy first of all sensitive, specific, reproducible, feasible and cheap diagnostic tests are needed (de Raadt 1977). Since the discovery of *Leishmania*, microscopic demonstration of *Leishmania donovani* (LD) bodies from spleen or bone marrow aspirate continues to be the recommended mode for confirmation of the diagnosis (WHO 1996). However -in addition to being an invasive test- microscopy is not feasible in the endemic countries where most peripheral hospitals lack laboratory facilities and/or trained personnel. Over the last 2 decades alternative serological tests for diagnosis of VL in the field, the direct agglutination test (DAT) (El Harith et al. 1986) and the immunochromatographic rK 39 test (based on 39-amino acid repeat recombinant leishmanial antigen from *Leishmania chagasi*) (Sundar et al. 1998) have been developed, but published estimates of their accuracy have varied.

The development of any new diagnostic test undergoes different phases of evaluation. As proposed by Zhou et al. (2002) in phase I, the exploratory phase, the objective is to assess whether the new test has any diagnostic value and comparison is made between

confirmed cases and healthy volunteers. Subsequently in phase II, challenge phase, the test's accuracy is challenged in potentially different subgroups of patients with and without the disease. As the study sample in phase II does not reflect the relative prevalence of the subpopulations from the target population it is usually not possible to measure the test's operational accuracy in this phase. This is overcome in phase III, the clinical phase, where the test is evaluated on a representative sample of patients from a well defined clinical population. A recent consensus statement on evaluation of diagnostics underlined the crucial need for properly conducted phase III trials (Whiting et al. 2004).

The DAT showed good performance in laboratory trials (Phase I and II) using banked sera (El Harith et al. 1986; el Safi et al. 1995; Singla et al. 1993; Sinha & Sehgal 1994), however its performance in the clinical setting (phase III) showed contradictory results with low specificity in some reports (el Masum et al. 1995; Zijlstra et al. 1991). Similarly the performance of rK 39 strip test showed variable results depending on the phase of study, location of the study and the brand of the dip-stick (Delgado et al. 2001; Schallig et al. 2002; Sundar et al. 1998; Veeken et al. 2003; Zijlstra et al. 2001). As few studies evaluated these tests on patients' representative for the clinical setting (phase III), there remained a need to more thoroughly evaluate these "easy to use rapid tests" before practice guidelines and policy recommendations could be formulated.

Finally, the arsenal available for treatment for VL is limited and the available drugs are either associated with significant toxicity (sodium stibogluconate, amphotericin B deoxycholate, pentamidine) or unaffordable (liposomal amphotericin B). Sodium stibogluconate (SSG), a pentavalent antimonial, which has been in use for over 6 decades, remains the 1st line therapy in most regions. Decreasing cure rates with SSG in the early 1980's were overcome by increasing the dose and duration of therapy. However, since the early 1990's reports from the adjoining Bihar state in India showed progressive decrease of cure rates even with the maximum recommended doses, reaching levels as low as 35% (Sundar et al. 2000) along with high case fatality rates

and cardiotoxicity (5 to 10%) (Singh et al. 1989; Sundar et al. 1997). There was and is little information available on the efficacy or toxicity of SSG in Nepal, though it continues to be recommended as a 1st line regimen in the national control programme. Hence, it was important to assess these parameters in Nepal in order to provide evidence for a rational drug policy for the control programme.

All the above led us to formulate the following objectives for the clinical and epidemiological research on VL which we launched in 1999.

2.2 Objectives

General objective:

Provide evidence for more rational VL control and case management of kala-azar in the Indian subcontinent in general and in Nepal in particular.

Specific objectives:

1. Describe the clinical and epidemiological pattern and the burden of disease due to kala-azar in Nepal
2. Evaluate the possible contribution to diagnosis of the recently developed diagnostic tests
3. Explore the efficacy and safety of currently used anti-kala-azar drugs.

2.3 Presentation of the published work

2.3.1 Clinical and epidemiological pattern and burden of disease due to kala-azar

The objective of the paper in chapter 3.1 ("Visceral leishmaniasis in Nepal: spreading endemicity and emerging antimony resistance") was to describe the extent and trend of visceral leishmaniasis in Nepal. A longitudinal description of 926 VL cases over a period

of 5.5 years (1999 to 2004) is given from the perspective of a referral hospital in an endemic region. It presents the demographic and clinical profile of patients presenting from the different districts of the country along with the duration of the illness and the outcome of the current treatment regimens. We show a trend of increasing number of cases from some non-endemic districts, which hitherto has not been picked up in the surveillance reports of the Ministry of Health. Also, in 2004 in comparison to 2003 there is a more than 2 fold increase in cases from Sunsari district and an over 10 fold increase from Dharan town, suggesting an emerging outbreak. Our findings also signal the possibility of further extension of the VL endemic to the previously not affected hilly districts.

In anthroponotic VL, where humans are the only reservoir, post kala-azar dermal leishmaniasis (PKDL) has been implicated for initiating (Addy & Nandy 1992) and sustaining epidemics. Such cases have been neglected by the control programmes (Thakur & Kumar 1992). PKDL is commonly seen in the clinical practice at BPKIHS (Garg et al. 2001). In the paper in chapter 3.2 ("Post-kala-azar dermal leishmaniasis with visceral leishmaniasis: a rare presentation") we document an unusual presentation of a case of PKDL combined with VL in a previously under-treated case of VL. This particular combination is estimated to occur in 1:700 PKDL cases (Sen Gupta PC 1968), and may reflect an important burden of PKDL in the community.

It has been known that VL patients in the Indian subcontinent come from the poorest socio-economic class (Thakur 2000), but the economic impact of the disease on the affected community has not been clearly elucidated. During a survey in a rural region, Charigua hamlet was identified as being amongst the most affected with VL. Information on all direct and indirect costs incurred in the households with VL, over the last 3 years, was collected. The results are reported in the paper "The economic burden of visceral leishmaniasis for households in Nepal" (chapter 3.3). In this hamlet, where almost 15% of the individuals had suffered from kala-azar during the past three years, the cost of the disease was enormous. Household expenditure incurred for a case of VL

was above the median annual per capita income and over 75 % of the direct costs related to treatment occurred even before VL treatment was actually obtained (for free). The ill equipped peripheral health care centres which lack tools to diagnose or treat VL prompted patients to first seek treatment with local faith healers or private practitioners, which contributed substantially to the incurred cost.

2.3.2. Evaluation of novel diagnostic tests

Recently serological tests, DAT and rK39 immunochromatographic test have been proposed as field tests but their performance was not always consistent. We thus conducted a prospective study presented in chapter 4.1 ("Prospective evaluation and comparison of the direct agglutination test and an rK 39 antigen-based strip test for the diagnosis of suspected kala-azar in Nepal") to validate these two tests using microscopy for LD bodies (bone marrow and/or spleen aspirate) as the reference test. The study was conducted at B.P. Koirala Institute of Health Sciences (BPKIHS), a university hospital in Dharan, eastern Nepal, on a cohort of 184 clinical VL suspect cases. Although the sensitivity of rK39 strip test was 97%, its specificity was evaluated at only 71%. For DAT the sensitivity was 99% with lowest cut off titer (1:400) and specificity did not exceed 82% even with a high cut off titer (1: 512,000). With these results these tests could only be recommended as a screening test. However, the specificity of the tests may have been underestimated due to the use of an imperfect reference test. Although microscopy for VL is very specific the sensitivity is below 90%, except in spleen aspirate, which was not possible to perform in all the cases due to contraindications. It has been shown that a flawed reference test can substantially affect validity estimates of new tests under scrutiny (Staquet et al. 1981). Hadgu and Qu (1998) suggested that latent class analysis (LCA), a mathematical modeling technique, which models associations between observed variables that imperfectly measure a non observable (latent) variable, could be a potential solution to overcome the gold standard problem. In the paper in

chapter 4.2 ("A comparative study of the effectiveness of diagnostic tests for visceral leishmaniasis"), we compared the validity of 4 serological tests, rK39 strip test, DAT, formol-gel test (FGT) and the indirect fluorescence antibody test along with pancytopenia as diagnostic criteria for visceral leishmaniasis on another cohort of 310 clinical suspect VL cases at BPKIHS. The sensitivity and specificity of all the tests were determined with parasitology as the reference test and also by applying LCA. For both the rK 39 strip test and DAT, the sensitivity estimates obtained by LCA were similar to the estimates with parasitology as reference test, but specificity estimates were substantially higher with LCA (DAT = 93.7% versus 77.8%; rK39 strip test = 93% versus 77%). In addition to confirming the doubts with regards to parasitology as a gold standard, the study provided evidence that the serological tests, DAT or the rK39 strip test, can replace parasitology as a diagnostic test for VL in the Indian subcontinent. The choice between the two would depend on other factors like the ease of use, cost and the availability.

The disadvantage of these serological tests is their inability to discriminate between clinical, subclinical or past VL infections. A urine leishmanin antigen detection agglutination test (KAtex) had been recently developed and evaluated in laboratory trials with encouraging results (Attar et al. 2001). As this could potentially overcome the disadvantage of the serological tests we decided to evaluate this test in the field which is presented in chapter 4.3 ("Evaluation of a urinary antigen-based latex agglutination test in the diagnosis of kala-azar in eastern Nepal"). The validity and reproducibility of KAtex was determined on 232 clinical suspects of VL using parasitology combined with DAT as the reference standard. It showed excellent specificity (98.7%) but the sensitivity was quite low (47.7%). The reproducibility, between the laboratories in Nepal and Antwerp, Belgium, was only moderate. Although this antigen detection test could become a real breakthrough in VL diagnosis there is a need to further improve its performance to become useful.

Even in the endemic countries the performance of any test in a peripheral level hospital may be quite different from that in a referral hospital due to various reasons. Firstly, patients in the district level may present with less advanced disease which may influence the sensitivity or specificity. Also the prevalence of VL in clinical suspects would be different from that of a referral level, thus influencing the predictive values of a test. Finally, the limited training of staff could affect the execution and/or interpretation of the tests. As most of the VL patients present in peripheral hospitals it was essential to know the usefulness of the VL tests at this level of care. Between the rK39 strip test and DAT, the former test is considered a simpler test to perform and interpret. We thus assessed the accuracy, reproducibility and feasibility of the rK39 strip test along with the FGT and the KAtex on 142 clinical suspect VL recruited at Rangeli district hospital, a peripheral level hospital, which is presented in chapter 4.4 ("Field validity and feasibility of diagnostic tests for visceral leishmaniasis in rural Nepal"). The reference test was based on a combination of parasitology along with DAT, the latter being performed at BPKIHS. The sensitivity of the rK39 strip test was 89% and significantly higher than FGT (52%) and KAtex (57%), though the specificity was over 90% in all three. The reproducibility of rK39 strip test was also found to be high and above the others. The study thus provided evidence for the feasibility of integration of the rK39 strip test as a diagnostic test in the peripheral level health services.

2.3.3 Efficacy and safety of current drug treatment

Pentavalent antimonials *e.g.* SSG, continues to be used, as a 1st line therapy, in most of the regions including Nepal. With reports of increasing failure rates to the drug from the adjoining Bihar focus in India, we decided to document the response of this drug in Nepal which is presented in chapter 5.1 ("Treatment of visceral leishmaniasis in south-eastern Nepal: decreasing efficacy of sodium stibogluconate and need for a policy to limit further decline"). One hundred and twenty new cases of VL were treated with the

full dose and duration of SSG (20 mg/kg/day for 30 days) and were followed up for another 6 months to asses the definite cure rate. Patients who failed their follow up appointments were traced in the villages. The overall definite cure rates, in those who completed the full therapy and follow up, was 90% but it was only 76% in patients who came from a district bordering the high resistance Bihar focus. These results provide evidence that the decreasing cure rate with SSG seen in Bihar, India, has extended to Nepal, which could be related to the spread of resistant strains.

In addition to the efficacy of SSG, its safety has also been a concern. Besides inherent toxicity of SSG, higher toxicity and deaths have also been related to the quality of the drug. In the paper "Sodium stibogluconate cardiotoxicity and safety of generics" (chapter 5.2), we report an outbreak of cardiotoxicity and deaths related to the use of a particular brand of generic SSG in Nepal in April/May 2000. In the 23 patients treated with this particular generic brand, there were 8 (35%) deaths which is in contrast to 3.2% deaths in the 252 patients treated, from August 1999 to December 2001, with the regular generic brand. Almost all the deaths were due to cardiotoxicity. Although generic versions of SSG have been shown to be equivalent to branded SSG (Moore et al. 2001; Ritmeijer et al. 2001; Veeken et al. 2000) it is important to ensure their safety and efficacy by applying rigorous quality control.

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CHAPTER 3:

CLINICAL & EPIDEMIOLOGICAL PATTERN AND BURDEN OF DISEASE DUE TO KALA-AZAR IN NEPAL

Title: Visceral Leishmaniasis in Nepal: spreading endemicity and emerging antimony resistance

(Submitted)

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Abstract: Visceral leishmaniasis (VL) is endemic in south eastern Nepal in spite of the control efforts. We document the epidemiological trend and treatment outcome of cases admitted from 1999 – 2004 to a teaching hospital situated in the endemic area. Over the whole period 926 VL cases were admitted from 13 districts, 4 of which are non-endemic for VL. In 2004 the cases, both from the rural and urban regions, increased by over 2 folds compared to 2003. In 1st time treated patients, cure rates (initial and definite) with the recommended 1st line drug, sodium stibogluconate (SSG), were significantly lower in cases from districts close to Bihar, the Indian high resistance focus (76.6% and 59.9% respectively) than in cases from other districts (92.5% and 86.1% respectively). Our study signals silent epidemics alongside extension of the VL endemicity in Nepal and evidences the spread of SSG resistance from Bihar.

Key words: visceral leishmaniasis; kala-azar; epidemiology; treatment; sodium stibogluconate; treatment failure; drug resistance; Nepal

Introduction

Visceral leishmaniasis (VL) is the most severe, systemic, form of leishmaniasis, and is almost always fatal if untreated (1). Although VL is reported from 61 countries, more than 90 % of the estimated annual 500,000 new cases occur in India, Bangladesh, Nepal, Sudan and Brazil (2). In the Indian subcontinent and Sudan VL is caused by anthroponotical transmission of *Leishmania donovani* and it typically affects the poorest people from the rural communities.

In Nepal, the first officially reported case of VL or kala-azar was observed in 1980. Since 1992, the VL incidence rate has been increasing rapidly, reaching up to 55/100,000 person-years in the past few years (3). More than 5.5 million people are estimated to be at risk of the disease and presently 12 districts in the southern plains or Terai (Nepal lowlands) region, bordering the state of Bihar, India, are endemic with a few more districts showing sporadic cases (4). The 1980 VL case was observed in Dhanusha district. Before that date, there is no historic record of VL in Nepal, but an Indian scientist who surveyed the region for vector borne diseases in 1949 claimed that Kala-azar was endemic in the entire Terai (5).

In India, there are records of major epidemics of VL in the eastern part since the first quarter of the nineteenth century, extracting a heavy toll on human lives (6). In the 1950's and 1960's there was a dramatic drop in the number of cases due to the effect of extensive DDT spraying in the malaria eradication programme. The last 2 epidemics in India had their origin in north Bihar, adjoining the Terai districts of eastern and central regions of Nepal. The most recent epidemic in Bihar, which started in 1991/1992, is estimated to have caused 200,000 to 250,000 cases (7). Most likely, the current epidemic in Nepal is a cross-border extension of this Bihar focus, as exemplified by the homogeneity of the *L.donovani* population (personal communication G.Schoenian).

The Ministry of Health (MOH) of Nepal launched, in 1992, a national programme for the integrated control of vector borne diseases, targeting malaria, kala-azar and visceral

leishmaniasis. This includes provision of free anti-VL drugs to district hospitals along with selective indoor residual insecticide spraying in VL endemic areas. Nevertheless, the VL case numbers continue to rise. In 1993 Nepal reported 1368 VL cases, but the more recent epidemiological surveillance figures show 2020, 2389 and 1921 new cases in 2001, 2002, and 2003 respectively (MOH, unpublished). It is believed that the reported VL figures are a gross underestimate of the true VL incidence, as many patients consult in the informal or private sector, and their records never end up in the surveillance data (4).

One of the major limitations for control of VL in Nepal has been the lack of adequate epidemiological data (4) apart from figures from 2 small studies. In 1996-1997, Koirala *et al*. (8) conducted a cross-sectional sero-survey in 2 villages in the eastern region, and showed a seropositivity of 6.09 % (n=1083) for *Leishmania* infection using the direct agglutination test. A case control study looking at risk factors for VL that included 84 cases and 105 controls from 3 districts from the central region concluded that sleeping on a bed or cot and sleeping under a bed net were protective against VL (9).

This paper provides the first longitudinal data from Nepal, over a period of 5 years (1999 to 2004), and reports the epidemiological and clinical profile of patients and on trends of VL as seen from a teaching hospital located in the heart of the endemic region.

Patients and Methods:

Context

Nepal is ecologically divided into 3 distinct regions: mountain in the north, hilly region in between, and the plains (Terai) in the south, bordering India. In the latter, a sub-tropical climate prevails, and it is affected by several tropical diseases. B.P. Koirala Institute of Health Sciences (BPKIHS) is a health sciences university, situated in Dharan, Sunsari district, in the Terai region (Figure 1). Its 640-bed teaching hospital serves as a referral hospital for the eastern region of Nepal, and the tropical disease unit is known to the

inhabitants of Terai as a specialized kala-azar centre providing free treatment to kala-azar patients. It also attracts a major part of the VL patients from the neighboring districts. Patients usually tend to seek care at BPKIHS after traditional healers or private practitioners have failed to cure their symptoms. Several others consult directly without going to the first line public health facilities.

Patients

The observations are based on a database of all patients diagnosed with VL who were admitted at BPKIHS from August 1999 to the end of 2004. The diagnosis of VL was suspected in all persons presenting with a history of fever of 2 weeks or more with a palpable spleen. The diagnosis of "confirmed VL" was made by demonstration of *Leishmania donovani* amastigotes in bone-marrow or spleen aspirate smears stained with Giemsa stain. Clinical suspects who were parasitologically negative but serology positive (rK39 immunochromatographic test or direct agglutination test titer, >1:3200 titer) and in whom other diseases were ruled out were considered as "probable VL". Age, sex, occupation, place of residence (district and village development corporation/town), duration of symptoms, clinical characteristics, haematological profile, treatment received and outcome were routinely recorded.

Treatment and follow up:

As per the national guidelines, confirmed and probable VL cases were treated with a pentavalent antimony (sodium stibogluconate (SSG), 20/mg/kg of body weight, intramuscular, for 30 days) as a first line therapy, except for one series of patients that accepted to be enrolled in a Phase IV study of Miltefosine between September 2003 and March 2004. Amphotericin B, 0.5-1mg/kg/day, infusion for 14 days was given as the second line therapy in cases of treatment failure. The patients received the full course of treatment either in the hospital or ambulatory. The latter applied only for the 1st line therapy in those who were unable to stay in the hospital for the full course. These

patients were supplied the full dose at the time of discharge, to be taken at the nearest health facility, with recording of the doses on a treatment card. All patients were followed up for clinical and parasitological evaluation at the end of treatment and at 6 months after treatment.

Outcome of treatment was defined as: initial cure, *i.e.* resolution of clinical features along with a negative parasitology at the end of treatment; definite cure, *i.e.* initial cure who did not show signs of relapse (recurrence of fever, increase in spleen size) during 6 months follow up; non-response, *i.e.* a person who remained parasitologically positive at the end of treatment; relapse, those with initial cure but recurrence of symptoms along with positive parasitology during a 6 months follow up period. Treatment failure was defined as either non-response or relapse. All patients were encouraged to present for a follow-up visit at 6 months. Reimbursement of transport cost and compensation for daily wage lost for this follow-up visit was available throughout most of the study period. The patients for whom no information was available after starting treatment, due to loss to follow up, were excluded from the analysis of outcome of treatment.

The data were analysed with EpiInfo 2002 (Centers for Disease Control and Prevention, Atlanta, GA) and SPSS v. 11 (SPSS, Chicago, IL).

Results:

A total of 926 VL cases were admitted between August 1999 and December 2004 at BPKIHS. The cases were admitted throughout the year with a higher proportion between March to July and the lowest in December and January. This pattern was similar every year.

Patients came from 13 districts (9 known endemic and 4 non-endemic ones), (Table 1) with the majority of the cases (87 %) from the 3 closest districts, namely, Morang, Sunsari and Saptari (Figure 1, Nº 2,3 &4 respectively). Interestingly, 23 patients (2.5 %) were admitted from the non-endemic districts Bhojpur (Nº 12), Dhankuta (Nº 11), Ilam (Nº

10) and Bardia (No 13), the former 3 being hilly and the latter a lowland district in far western Nepal.

In 2004 there was a dramatic increase in the number of cases when compared to the preceding years, both from non endemic and endemic districts (Table 2). There were in particular 9 cases from Bhojpur (No 12). In Sunsari district, where BPKIHS is located, the number of admissions from the rural areas (village development corporations) almost tripled in the last year. In Dharan town there was a dramatic, almost 10-fold, increase in the number of cases in 2004.

Ninety percent of the total cases were confirmed VL and 10% probable VL, 56% were males and age-wise 29.5 %, 60.9% and 9.6% were less than 15 years, 15 to 45 years and over 45 years respectively. The duration of history of fever prior to admission ranged from 2 to 104 weeks with a median of 8 weeks (Inter quartile range 4-12).In cases from the non-endemic districts the proportion of males and the duration of fever prior to admission was higher than in cases from the endemic districts, and none of them had received past treatment for VL (Table 1).

Overall, 125 out of the 926 patients (13.5%) had history of treatment of VL in the past. Majority of these had received SSG (76.8%) and 80% had given history of receiving the full course (although there was no documents available with most of them). While 80% had received the treatment in a public health facility only 20 % received it at 1st line health services. The incidence of co-infection with HIV was nil from 1999 to 2002, and 0.8 % and 0.75 % in 2003 and 2004 respectively. All HIV positive cases were from the urban regions.

On admission at BPKIHS, 823 patients were treated with the 1st line therapy - 700 with sodium stibogluconate (Albert David, Calcutta, India) and 123 with miltefosine (Zentaris, Frankfurt, Germany) - and 92 with 2nd line therapy Ampotericin B. Six patients defaulted and 5 died before the specific treatment could be started. In those who were treated the follow up visit at end of treatment and at 6 months was achieved in 92 % and 60% respectively. Outcome of treatment in 1st time SSG treated patients is shown in table 3. Both the initial and definite cure rate was significantly lower, by 15.8 % and 26.5%

respectively, in cases coming from the districts bordering the Bihar high resistance zone (figure 1: district nº 4 to 9). Also the death rate and toxicity to SSG was higher in cases from these districts. The highest death rate was noted in the year 2000, when it was 13.5 % in patients from districts bordering the Bihar high resistance zone and 6.9% in the other patients.

Discussion:

As our data show, VL has become well established in the Nepal plains as a major endemic tropical disease. Although the majority of the patients are still from the lowland districts, there is an alarming trend in the number of cases from the non endemic hilly districts. Curiously, there have been no records of cases from these districts in the surveillance reports of MOH from 1999 to 2003. The increase we observed at BPKIHS signals, in our view, the appearance of new foci in Bhojpur, Ilam and Dhankuta districts, as there was no history of recent travel to known endemic regions in the cases originating there.

Moreover, as the national VL control programme does not cover these districts, it is likely that several cases of VL are not diagnosed or treated in the local hospitals, and the number of cases seen in this cohort may well represent only a fraction of the total cases occurring in the hilly areas. The longer duration of fever, which is an approximation of the duration of the disease, in these cases reflects the problems of access to our center. The higher proportion of males is also related to the difficulty, especially for women, to come to BPKIHS, which requires a few days of travel. The single case from Bardia district (Nº 13) in far western Nepal traveled over 600 kms, as he could not obtain the anti VL drugs locally. He gave a history of travel to north India before the start of the illness. Migration to other countries, including India, for work has generally increased recently due to the ongoing violent conflict and increasing poverty, and may be an important risk factor for the occurrence of the disease in the non endemic regions.

A limitation of this study is that our data are hospital based, and we cannot absolutely exclude that the increasing trend we observed might be due to higher attraction of cases

to the hospital in comparison to the previous years. The doubling of cases in 2004, compared to 2003, could be for instance related to the spreading news of an oral drug, Miltefosine, becoming available at our centre.(10). However, this cannot be a factor of meaningful importance for patients from Sunsari district, where BPKIHS is located. Those patients traditionally seek treatment at BPKIHS, which is easily accessible for them and has been providing free treatment to VL patients during the whole study period. Therefore, the dramatic increase of the number of cases from Sunsari district in 2004, suggests in our view an increasing transmission of the disease. The figures for Dharan and Itahari, both urban areas within Sunsari district, seem even to indicate an evolving outbreak. Almost all the cases in these towns occurred in recent suburban settlements, which are unorganized with poor environmental conditions. Migration from rural to urban areas has also in Brazil led to quick urbanization of VL (11). The urbanization of VL in this region of the world carries the threat of rapid increase of VL and HIV co-infection, which at present appears to be still quite low. However, towns like Dharan are known to have a high prevalence of intravenous drug users and recent reports found a high prevalence of HIV infection (45 to 60%) in these groups (12).

The case fatality rate (CFR) in patients treated with SSG in our study is higher than what has been reported by the epidemiological surveillance system of MOH (around 1%), but we feel ours is probably a more realistic rate. Indeed, in the public health facilities follow up information after completion of treatment is available in only a few patients. Though it has been felt that SSG at the present recommended doses is considered safe (13), this mainly refers to patients treated for cutaneous or mucocutaneous leishmaniasis. Reports of higher CFR in visceral leishmaniasis treated with SSG have been noted elsewhere 5.9% in India (14)and 8.4% in Sudan (15). In addition to the failure to respond, cardiotoxicity due to SSG is known to increase the mortality and this is related to the quality of the SSG. A high rate of cardiotoxicity and deaths occurred in our hospital in the year 2000 linked to the use of one batch of SSG produced by a particular generic manufacturer (16). Similar incidents have also been reported from India (17).

Although the pentavalent antimonials have been in use for more than 50 years, they continue to remain the first line therapy in most countries, including Nepal. This is mainly attributable to it's time tested efficacy and affordability. However, over the last decade there have been consistent reports of progressive decrease of the cure rates with SSG in Bihar, India (18). Estimated cure rates as low as 35 % have led to abandoning their use as a first line agent (14;19;20).The present study documents, for patients treated for the first time with SSG, significantly lower cure rates in those cases coming from the districts bordering the high resistance Bihar focus. This confirms an earlier observation where cases from Saptari district (No 4) had a definite cure rate of only 76% when compared to the overall cure rate of 90% in a cohort of novel patients from Morang, Sunsari and Saptari treated with SSG (21). Important reasons for treatment failure have included rampant use of sub therapeutic doses and incomplete duration of treatment which are likely to be the main determinants of the emergence of resistant strains of *Leishmania donovani* to SSG, which have been isolated from relapsed patients in Bihar (22). In our study the majority of our patients that have been treated in the past had received complete courses, which is in contrast to studies from Bihar where only 26% of cases presenting with refractory disease had received treatment as per the WHO guidelines (23;24).The close proximity of Bihar, along with the intense cross border movement, in addition to increased transmission, could easily explain the spread of drug resistance from Bihar to Nepal.

In spite of the control measures implemented by the national programme, which has been operational for over 10 years, we provide evidence of intensified transmission of VL in Nepal and of the emergence of new foci. The surveillance system and the national programme need to be strengthened and extended to these "non endemic districts" where cases are appearing. Epidemiological and entomological surveys should be conducted to better document the emergence of new epidemic foci. Recommendations for the use of SSG as a first line therapy in the national programme needs to be revised urgently, particularly in those districts bordering the high resistance Bihar focus. The epidemiological trends in Nepal, including the spread of resistance to drugs, are being

heavily influenced by the Indian epidemic and a close cross border collaboration between the control programs of the two countries is essential. The recently launched kala-azar elimination programme for the region would be a good platform to build this collaboration (25)

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Table 1: Origin and profile of VL cases admitted at BPKIHS, Nepal, August 1999 to December 2004

District	Nº on map	Endemic	Nº of cases	% males	% < 15 years	Median duration of fever (weeks)	% Past treatment for VL
Sunsari	3	Yes	355	53.5	33.9	8	4.8
Morang	2	Yes	268	57.1	33.2	6	10.1
Saptari	4	Yes	183	52.5	33.3	6	30.1
Siraha	6	Yes	34	70.6	38.2	4	20.6
Jhapa	1	Yes	24	62.5	29.2	5	12.5
Bhojpur	12	No	17	76.5	29.4	18	0
Dhanusha	7	Yes	14	71.4	14.3	8	35.7
Sarlahi	9	Yes	12	58.3	8.3	14	50
Mohottari	8	Yes	7	42.9	28.6	7	14.3
Udayapur	5	Yes	6	50	16.7	6	16.7
Dhankuta	11	No	3	66.7	0	24	0
Ilam	10	No	2	100	0	9	0
Bardia	13	No	1	100	0	13	0
All	-	-	926	56	29.6	8	13.2

Table 2: Yearly number of VL cases admitted at BPKIHS, Nepal by endemicity of districts of origin and by rural or urban regions from Sunsari District, 2000 to 2004

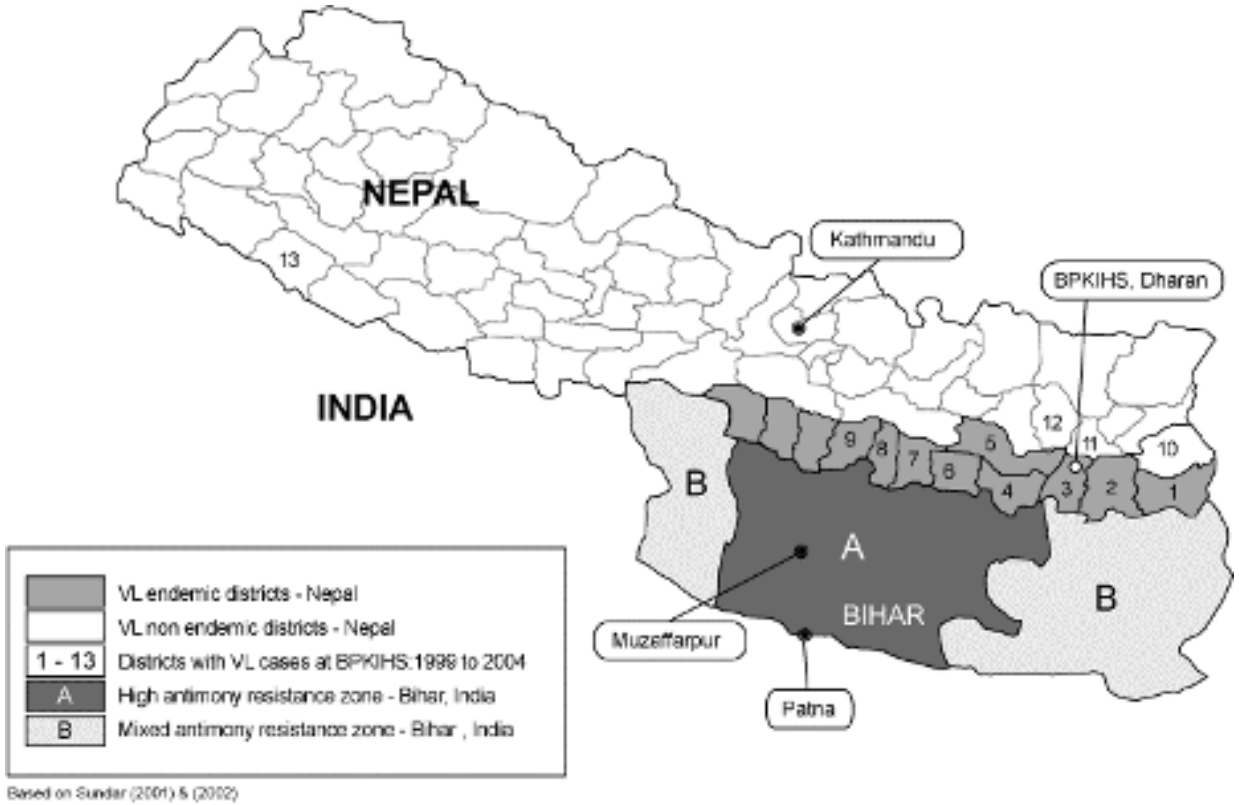
	2000	2001	2002	2003	2004
All districts					
Endemic	155	148	143	122	271
Non endemic	2	2	5	2	12
Total	**157**	**150**	**148**	**124**	**283**
Sunsari district					
Rural regions					
Amahibelaha	1	2	0	2	13
Aurabani	1	0	2	0	17
Barahachetra	6	6	8	1	2
Bokraha	0	2	0	5	17
Others	37	23	27	21	32
Sub total	**45**	**33**	**37**	**29**	**81**
Urban regions					
Dharan	6	9	7	3	44
Inaruwa	2	2	3	8	1
Itahari	2	0	10	9	4
Sub total	**10**	**11**	**20**	**20**	**49**
Total (Sunsari)	**55**	**44**	**57**	**49**	**130**

Table 3: Outcome of treatment with sodium stibogluconate in treatment naïve cases, BPKIHS, Nepal, 1999 to 2004.

	No. treated	No. assessed	% Initial cure	% Treatment failure*	% Death	% Toxicity #	% Definite cure**	% Lost to follow up
Districts away from resistant Bihar focus	526	480	92.5	4.5	2.7	1.3	86.1	8.7
Districts bordering high resistant Bihar focus	133	133	76.6	14.3	6.8	4.5	59.9	10.7
Difference (95% CI)	-	-	15.8 (8.2 to 23.4)	-9.7 (-15.9 to -3.5)	-4.1 (-8.6 to 0.5)	-3.3 (-6.9 to 0.4)	26.5 (15.3 to 37.7)	-2.0 (-7.5 to 3.5)

* Includes both non-response and relapse.
# Toxicity requiring change of treatment
** Definite cure rate assessed in 293 cases from districts away and 84 cases from districts bordering high resistance focus.

Figure1: Visceral leishmaniasis endemic districts in Nepal, districts with VL cases attending BPKIHS, Nepal (1999 to 2004) and antimony resistant zones in Bihar, India.



Tropical medicine rounds

Post-kala-azar dermal leishmaniasis with visceral leishmaniasis: a rare presentation

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Case Report

A 48-year-old man presented with multiple papules, nodules and verrucous plaques on the face, trunk and extremities of 1-year duration. Two years ago he had prolonged fever and splenomegaly and was diagnosed with kala-azar, for which he received inadequate treatment from the local health center. A year later he developed erythematous papules in the perioral region and these lesions gradually increased in size and number. Simultaneously he also developed multiple nodules in the extremities along with hypopigmented macules on the trunk. A diagnosis of lepromatous leprosy was made at the local health center and he was started on World Health Organization-multi bacillary multi drug therapy (WHO-MBMDT). He discontinued the medication after 6 months when there was no improvement in his lesions.

There was no history of hypoaesthesia over the lesions, neuritis, epistaxis, pedal edema, eye complaints, orchitis, fever, loss of weight or loss of appetite.

On cutaneous examination he was found to have multiple hypopigmented macules, erythematous papules, nodules and plaques of various sizes on the face, trunk and extremities (Fig. 1). The plaques on the dorsum of the legs were large and verrucous with maceration of the skin (Fig. 2). Papules and nodules were also present on the dorsum of the tongue (Fig. 3), the hard palate and the scrotum. All the lesions were asymptomatic. No nerves were thickened or enlarged. He had multiple bilateral cervical and inguinal lymphadenopathy. The lymph nodes were 3–4 cm in size, discrete, nontender, mobile and firm. The skin over the nodes was normal. On systemic examination the spleen was found to be enlarged to 3 cm below left costal margin, and there was no hepatomegaly.

Figure 1 Nodules on the lips, nose, forehead and cheeks

The results of investigations were as follows: Hemoglobin was 9.2 g/dl and erythrocyte sedimentation rate (ESR) was 48 mm in 1 h. Urine microscopy, liver function tests, renal function tests, chest X-ray and electrocardiogram (ECG) were all within normal limits. The Mantoux test and human immunodeficiency virus (HIV) test were negative. Peripheral blood examination did not reveal the malaria parasite. Skin slit smears from the nodules, macules and plaques on the face, trunk, foot, leg and ear lobes for *Mycobacteriuum leprae* were negative. However, many *Leishmania donovani* bodies (LDB) were seen in these lesions stained with Giemsa. Fine needle aspiration cytology from the lesions and lymph nodes showed epitheloid cell granulomas with LDB. Skin biopsy from the skin lesions showed epitheloid cell granulomas with dense inflammatory infiltrate of plasma cells, histocytes

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Figure 2 Verrucous plaques on the dorsum of both feet

Figure 3 Nodular lesions on the tongue

and lymphocytes. A few LDB were also seen (Fig. 4). Bone marrow aspirate stained with Giemsa also showed many LDB and the rK39 dipstick test was found to be positive. The patient was diagnosed as a case of post-kala-azar dermal leishmaniasis with visceral leishmaniasis and was referred to the nearest primary health center with a prescription to start injections of sodium stibogluconate 20 mg/kg/day intramuscularly until all the lesions resolved.

Discussion

Post-kala-azar dermal leishmaniasis (PKDL) is a known squaelae to visceral leishmaniasis (VL) or kala-azar (KA) that develops in 10–20% patients in the Indian subcontinent. It usually manifests several months to a few years after "cure" of KA. However, in Sudan, it develops in nearly 56% of cured KA patients within weeks to a few months after treatment.[1]

The rash of PKDL may be macular, maculopapular, nodular or plaque-like. The lesions may become verrucous, papillo-

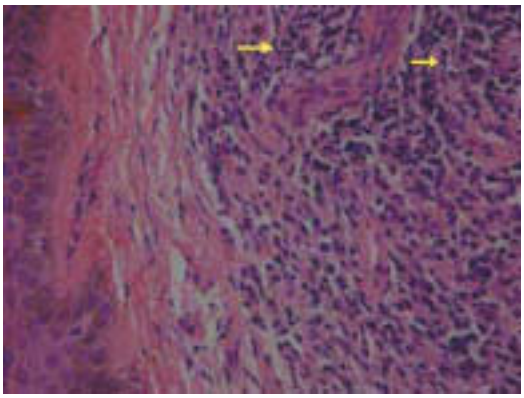

Figure 4 Microphotograph showing epitheloid cell granulomas with dense inflammatory infiltrates of plasma cells, histiocytes and lymphocytes in the dermis. A few LDB were also present and are marked by arrows (hematoxylin and eosin, ×40)

matous, annular or fibroid.[2] Ulceration is uncommon.[3] The lesions initially appear in the peri-oral region and then spread to the face, trunk and upper/lower extremities. Papules or nodules may be present on the scrotum. Mucosal lesions on the tongue are also uncommon.[4] Dissemination of the lesions all over the body may mimic lepromatous leprosy.[3] Lymph nodes may be enlarged but most PKDL patients do not have demonstrable parasites in lymph node or bone marrow aspiration.[1]

Our patient presented with large verrucous plaques, which is a rare presentation in PKDL, along with nodules on the tongue, face and upper limbs. The patient was misdiagnosed as having leprosy and received treatment for 6 months. Differentiation from leprosy is difficult in places where both diseases are endemic. However, diagnosis of PKDL can be made clinically provided that the appearance, distribution and temporal relationship with kala-azar are taken into account.[5] Diagnosis can be supported by slit skin smear examination for LDB. Therefore, it is important that physicians be aware of the differential diagnosis of PKDL.

The exact pathogenesis of PKDL is not known. However, there is evidence that immune responses have a major role. A high concentration of interleukin-10 (IL-10) in the peripheral blood of KA patients predicts the development of PKDL. After treatment of KA, peripheral blood mononuclear cells (PBMC) start producing IL-10, which plays an important role in the regulation of the immune system.[2] The immune system shifts from a T helper type 2 (Th2) to T helper type 1 (Th1) or to a mixed Th2/Th1 response. The Th1 cells migrate to the skin, and their subsequent attack on the parasites may be an important factor in the pathogenesis of PKDL.[3] The production of IL-10 by PBMC also coincides with the appearance of PKDL lesions.[2] Immunity after cure of KA has been known to be almost

lifelong, provided there is no immunosuppression.[5] The structural and functional component(s) of the visceral immunity may protect the host from recurrence of KA by preventing infection from freshly infected sandfly bites, or by blocking re-invasions of the parasites from the skin in the case of PKDL or by preventing reactivation of the latent parasites in the viscera.[5] However, a few cases of PKDL associated with KA have been reported in the literature.[5–12] One study from India estimated this to occur in one in every 700 patients with PKDL. A degree of immunosuppression induced by intercurrent diseases such as measles, malaria, tuberculosis and HIV infections favors reinvasion of the parasites from the skin to the viscera.[10]

The development of partial immunity after incomplete treatment leads to clinical improvement, but persistence of parasites seems likely. Failure to develop complete immunity may result in re-establishment of VL from the skin or from other organs. Renewed multiplication of the latent parasites in the viscera might be the cause of relapsed VL in our patient, as he received inadequate treatment for VL. However, re-infection as a cause of relapse of VL could not be ruled out because the patient was from an endemic area and the long interval between VL and PKDL leaves room for speculation about re-infection. We report this case because of its uncommon features and its rare association, and to point out that patients with long-standing unrecognized PKDL may play a role in transmission as they may act as a reservoir, particularly in India and Nepal where the transmission of KA is anthroponotic.

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THE ECONOMIC BURDEN OF VISCERAL LEISHMANIASIS FOR HOUSEHOLDS IN NEPAL (In Press: Transactions of the Royal Society of Tropical Medicine and Hygiene)

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Running title: Economic burden of VL in Nepal

SUMMARY

Visceral Leishmaniasis (VL) affects persons from the lowest socio-economic strata of the community, but its economic impact is not precisely known. An exploratory survey to document the economic costs of VL to households was conducted in an endemic focus in eastern Nepal and data was collected from the 20 households of this cluster. Cases of VL over the last 3 years were elicited and information on direct and indirect cost incurred due to the disease and income of the households over the last year was estimated. 15 % (16/107) of the residents had suffered from VL and almost all the patients had preferred, at the first instance, to visit the private services or local faith healers instead of visiting the local public health facility. Average total cost incurred per episode of VL was above the median annual per capita income and 6 of the 7 affected households had to either sell part of their livestock or take loan to cover the costs. Direct costs consisted of 53% of the total cost with 75 % of this cost incurred before the patients actually received any treatment for VL.

This study demonstrates how VL can lead to catastrophic expenditure for the affected households.

Keywords economic burden, visceral leishmaniasis, Nepal, poverty, health care

INTRODUCTION

Visceral leishmaniasis (VL), a vector-borne disease that is fatal if untreated, has recently earned public attention as one of the "most-neglected diseases" (Trouiller et al. 2002). The World Health Organization estimates that there are about 500,000 new VL cases per year, of which more than 90 % occur in India, Bangladesh, Nepal and Sudan, and 59,000 deaths (UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases 1997). However, the reported figures may be grossly underestimated. In Bihar, the epicenter of the Indian epidemic, door to door surveys revealed that the real prevalence was 5 times higher than the officially reported one (Desjeux 1991). Similarly, the Ministry of Health (MOH) of Nepal acknowledges that only those cases who attend the government health facilities are represented in the reported figures (Bista 1998). Moreover, as VL has a very focal distribution, the aggregated figures do not reflect the real burden of disease in affected communities. In prospective cohort studies reported incidence rates of VL vary between 2/1000 over 14/1000 person-years in Ethiopia (Ali and Ashford 1994), to 40/1000 person-year in Sudan (Zijlstra et al. 1994). Incidentally, these rates are comparable to or higher than the incidence rates of tuberculosis at community level.

VL is a poverty-related disease that affects the poorest of the poor (Desjeux 1996; Boelaert et al. 2000) . In Bihar, 75% of VL patients had a daily income < US$ 1 and 77% were living in mud or grass covered houses (Thakur 2000) .The vector, *Phlebotomous argentipes*, breeds in cracks of mud-plastered houses and moist soils. In Nepal almost all VL patients live in remote rural communities.

The impact of VL on economic development is still not fully recognized. Without quantifying it, Wijeyaratne et al. (1994) have drawn attention to the tremendous barrier the disease poses to the development of the affected communities. There is also little known on the possibly catastrophic economic consequences of VL on the affected households. Adhikari and Maskay (2003) in a letter to the editor made reference to the total medical cost incurred (US$ 210) per episode of VL care in Nepal and a qualitative survey on the perception of VL at community level in Bangladesh reported in passing how gathering the US$ 85 to 500 necessary for diagnosis and treatment left the household destitute (Ahluwalia et al. 2003). Moreover, in every family where a VL case occurs, many days of productive life may be lost directly or indirectly but this has never been quantified. We therefore conducted an exploratory study to better document the economic costs of VL to households.

METHODS

During a survey conducted in eastern Nepal to estimate the incidence of leishmanial infection and disease, Charigua, a hamlet in Dulari village, was identified to be amongst the most affected locality (unpublished data). VL being a focal disease with cases occurring in clusters, we selected this hamlet which would be representative of other VL foci within the region. Charigua hamlet consisted of 20 huts, between the village road and the paddy fields and half a kilometre away from the national highway. All the huts in Charigua are mud plastered with thatched roofs. A few families rear animals on their backyard, close to their huts. The nearest health facility, a sub-health post, was 2 kms away. This is the lowest level in the Nepalese public health system and supervised by an auxiliary health worker with 1 year training. A

district hospital run by 2 medical doctors and catering to a population of about 700,000 is located 20 kms away. A tertiary care university hospital, B.P. Koirala Institute of Health Sciences (BPKIHS) is situated 25 kms away, and is easily accessible by bus or taxi via the above mentioned highway. BPKIHS has been providing free VL care (VL drugs, diagnostic tests and hospitalisation fees) to patients over the last 5 years.

From April to June 2004 every household in Charigua was visited by a trained interviewer. Data was collected from the head of the household and the patients and/or their caretakers in case of children on a pre-tested questionnaire. First, VL episodes (which was defined as fever treated with VL drugs) occurring in the household in the last 3 years were elicited, and these data were later cross-checked and validated against the hospital admission records at BPKIHS. For all episodes health seeking behavior was recalled including local faith healers, private services (pharmacies and private practitioners) and public health services. Information collected on costs included direct health care cost (diagnostic tests, all drugs, fees paid to providers) and other associated cost (transportation and food) and indirect costs (earnings lost/foregone due to the illness by patients or caretakers). Costs incurred at the level of 1st line care (faith healers, private services and peripheral public health service) and at the level of referral level (BPKIHS) were separated. For all households we estimated the total yearly income from wages, agricultural activities and other sources in the past year. As the yearly inflation rate was low (around 5%), no adjustment for inflation was made. All costs were recorded in Nepalese rupees and have been transformed to US$ at the 2004 exchange rate (1US$= NRs 74).

RESULTS

The Charigua hamlet had a total population of 107, living in 20 households. The sex ratio was around 1:1 for both adults and children. Eighty percent was less than 45 years of age and children < 15 years comprised 42%. None of the households owned any farming land, and all the male adults were daily wage laborers, either farm laborers or rickshaw drivers. Some of the female household members and older children also worked occasionally, as laborers, mainly during the paddy plantation and harvest season. Livestock raised (and/or backyard gardening) was strictly for self consumption except during times of crises when they sold part of their livestock. The median annual per capita household income (PCI) was US$ 81.07 (Q1 69.44; Q3 113.47).

Over the past 3 years, 15 % (16/107) of the residents of Charigua had been diagnosed with VL. The cases were concentrated in 7 out of the 20 households. Six patients had first visited local faith healers, 9 private health services, and only one person had first consulted the public first-line health post. Fifteen patients eventually attended BPKIHS for VL treatment after a median delay of 8 weeks (Q1 4; Q3 12) and one received treatment in a private clinic. The median age of the patients was 23.5 years (Q1 11.25; Q3 30.75), of which 5 were < 15 years of age. All the patients were treated with sodium stibogluconate (20mg/kg/day for 28 days), the recommended first line therapy.

Notwithstanding the free provision of VL care at BPKIHS, the total cost incurred by patients was on average US$ 113.65 (median US $ 84.41 (Q1 50.09; Q3 139.50)) per episode (Table 1) which was above the median annual PCI of US$ 81.07. The median

cost of treatment for VL in those households which had more than one case (4 households) was US$ 425 (Q1 250; Q3 506) which is more than the median annual household income of US$ 405 (Q1 324; Q3 486) for the affected households.

Direct costs on average consisted of only 53% of the total costs. The indirect costs were due to loss of earnings for days lost due to illness or caretaker which was on average 57 days (median 37 days (Q1 13; Q3 75)). Of the total cost incurred at the 1st line care, 88.5% occurred in the private health services and only 11.5 % during visits to the local faith healers.

The direct costs incurred in the 1st line or referral level care were comparable, but in the former it mainly consisted of health care cost (89%) and in the latter, other associated costs (66%) which were essentially for transport and food for the patient and their caretakers. Seventy five percent of the mean total direct health care cost occurred at the 1st line care before the patient had actually received any VL treatment.

All 7 affected households used their savings and also borrowed money from their friends and neighbors to cover the cost of treatment. Additionally, three households had to take a loan from money lenders at very high interest rates (6% per month), and 3 other households had to sell part of their livestock assets.

DISCUSSION

This exploratory study quantifies for the first time the different components contributing to economic cost incurred due to VL and shows how VL can lead to enormous health expenditure at the household level. The 3 year recall used in our study is quite long for obtaining an accurate estimate of the expenses made, but as

these expenses had been quite substantial to these households and with little variation over this period, we feel our data are a good approximation of the costs incurred.

It is of note that, almost all patients first sought care from the local faith healers or private services although a public health post was within walking distance (2 km). Communities lack confidence in the health post which is equipped with limited facilities and drug supply. Surprisingly, most of the health care cost for VL care was incurred during the first line of care, which in this case was mainly in the private sector. In Nepal the private services in the rural areas, mainly consist of over-the counter sales of drugs in the medicine shops or poorly trained private practitioners with very irrational prescribing practices. There is no system of monitoring or regulation of this private sector. As with other neglected tropical diseases, VL can lead to "iatrogenic poverty" (Van Damme et al. 2004;Meessen et al. 2003).

The MOH in Nepal is committed to the control of kala-azar. The kala-azar control programme launched in 1992 has been providing free VL drugs to the public hospitals in the endemic region. However, as the diagnosis of kala-azar is only possible at the level of the district hospitals it appears that many VL patients are not able to get access to this "free care". The cost of travel and the loss of earnings deter most patients, who are daily wage laborers, to travel to these public hospitals at the first instance. Only 10.2 % of the population in rural Nepal lives within one hour travel to a public district hospital (Hotchkiss et al. 1998).

A survey conducted in India on the mean household expenditure related to an episode of kala-azar, observed a significant difference between the state of Bihar (US $ 401.6)

and West Bengal (US$ 59.8) (V Annigeri, unpublished report). These differences could be related to care seeking behavior of the community and the accessibility to the health system, the latter being much better developed in West Bengal compared to Bihar.

Our study indicates that quality VL treatment in Nepal is too centralized in specialized care centers situated in the larger towns and cities. The MOH should stimulate decentralization by introducing simple case-management strategies and by making reliable and feasible diagnostic tools eg. rK 39 dipstick test (Sundar 1998; Boelaert 2004), and drugs available in the peripheral levels of the health system. In addition to the provision of free VL care, activities to increase awareness within the community needs to be intensified. Koirala et al. (1998) observed poor knowledge of VL in the community, in a survey conducted in an endemic area in Eastern Nepal.

Most importantly, policy makers should recognize how neglected diseases such as VL can maintain the vicious circle of poverty, and should combat them more intensively including the strengthening of first-line health systems.

Conflicts of interest**:** The authors have no conflicts of interest concerning the work reported in this paper**.**

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Table 1.Distribution of the costs (US$) incurred for treatment of visceral leishmaniasis (n= 16) in Charigua, Nepal,2004

	Direct costs									Indirect costs	Total costs
	1st line care			Referral level care			Subtotal				
	Health care costs	Other costs	Both costs	Health care costs	Other costs	Both costs	Health care costs	Other costs	Both costs		
Mean	28.28	3.32	31.60	9.52	18.74	28.26	37.81	22.07	59.87	53.78	113.65
Median	8.34	1.6	9.87	4.17	19.59	22.86	10.11	21.61	29.19	39.96	84.41
Q1	2.78	0.56	3.51	1.5	7.54	12.32	6.43	9.97	26.69	15.12	50.09
Q3	51.56	4.72	54.14	6.34	24.36	28.44	56.94	24.64	82.17	94.87	139.50

CHAPTER 4:

EVALUATION OF NOVEL DIAGNOSTIC TESTS

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Prospective evaluation and comparison of the direct agglutination test and an rK39-antigen-based dipstick test for the diagnosis of suspected kala-azar in Nepal

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Summary The diagnosis of visceral leishmaniasis (kala-azar) remains difficult in rural endemic areas and practical and reliable tests are badly needed. Two serological tests, the Direct Agglutination Test (DAT) and an rK39-antigen-based dipstick test, were compared to parasitological diagnosis in a group of 184 patients presenting at a tertiary care centre in south-eastern Nepal with a history of fever ≥14 days and splenomegaly; 139 patients had a parasitologically proven kala-azar and 45 patients had a negative parasitological work-up. The rK39 dipstick showed a sensitivity of 97% and a specificity of 71%. The DAT was up to 99% sensitive with a low cut-off titre (1:400) but its specificity did not exceed 82% even with a high cut-off titre (1:51 200). Both tests could be used for screening suspect patients in endemic areas. However, their use as confirmatory tests should be restricted to situations where the proportion of kala-azar among clinical suspect patients is high. The rK39 dipstick is cheaper and easier to use than the DAT and could be used widely provided that both its performance and production remain stable.

keywords visceral leishmaniasis, kala-azar, diagnosis, direct agglutination test, k39 antigen, dipstick, Nepal

Introduction

An estimated 500 000 persons are affected by visceral leishmaniasis (VL, also known as kala-azar) every year worldwide. The vast majority of these cases (90%) occurs in poor rural areas of Sudan, India, Bangladesh, Nepal and Brazil (Desjeux 1996). Kala-azar is usually fatal if left untreated but the use of sodium stibogluconate, the most frequently prescribed first-line therapy generally considered to be relatively safe, can have severe side-effects (Sundar *et al.* 2000). It is, therefore, of prime importance to have accurate and practical diagnostic methods available in endemic areas. In most countries, diagnosis relies on microscopic examination of lymph nodes, bone marrow or spleen aspirates. Lymph node and bone marrow aspirations are safe procedures but their sensitivity for diagnosing kala-azar is only 58–64% and 70–86%, respectively (WHO 1984; Zijlstra *et al.* 1992). Spleen aspiration is generally considered as the gold standard for kala-azar diagnosis (Ho *et al.* 1948; Zijlstra *et al.* 1992) because of its high sensitivity and specificity (both close to 100%). However, it is contra-indicated in quite a number of kala-azar suspect patients, although it is a safer procedure than thought by many physicians. When performed properly, the death rate due to severe bleeding is 0.1% as reviewed by Kager & Rees (1983). One of the major drawbacks of parasitological diagnosis is the expertise required from both the physician to perform the procedures and the laboratory technician to stain and read the slides accurately. This expertise is very difficult to obtain in practice outside reference tertiary hospitals or specialized treatment or research centres.

Research has focused on the development of cheap, simple and reliable serological tests for kala-azar, which could replace parasitological diagnosis in the field. The Direct Agglutination Test (DAT) was first described by Allain & Kagan (1975) and the method was then adapted by El Harith *et al.* (1986, 1988). The DAT proved to be a

very valuable tool for field workers especially when high numbers of patients present to health facilities, as during the disastrous epidemics raging in Sudan for the last 15 years. The DAT is very accurate under laboratory conditions (El Safi & Evans 1989; Singla *et al.* 1993; Joshi *et al.* 1999) with high specificity when control groups were composed of healthy persons from endemic areas (Schaefer *et al.* 1995; Boelaert *et al.* 1999a). In field conditions, the DAT is very sensitive, but low specificity values were reported in series of clinical suspect patients, with a range depending on the reference test used, between 58% (Boelaert *et al.* 1999b) and 72% (Zijlstra *et al* 1991). Use of the DAT has been encouraged by WHO for surveillance and control programmes of VL (DAT workshop, Antwerp, 25–27 March 1998).

More recently, serological testing against a recombinant antigen derived from a 39-amino acid repeat in *Leishmania chagasi* (rK39) was developed. It is very accurate when used in an ELISA format (Singh *et al.* 1995; Badaro *et al.* 1996; Zijlstra *et al.* 1998). It was later developed as a dipstick format that showed to be 100% sensitive and 98% specific in India (Sundar *et al.* 1998). As Zijlstra *et al.* (2001) found a sensitivity of only 67% in Sudan these results require further confirmation.

Within this context of new diagnostic development, this study aimed at evaluating and comparing the validity of the DAT and an rK39-antigen-based dipstick for the diagnosis of VL among clinically suspect patients in Nepal.

Materials and methods

Study site

This study took place at the B.P. Koirala Institute of Health Sciences (BPKIHS), a 650-bed University Hospital located 2 km away from the town of Dharan, Sunsari District, Eastern Region of Nepal. The BPKIHS serves as a reference tertiary hospital for the eastern region, which includes several kala-azar endemic districts. Recruitment of patients took place in the Outpatient Department (OPD) and the Emergency Room of BPKIHS from July 1999 to August 2000. The research protocol was approved by the Ethical Committee of BPKIHS on May 1999.

Inclusion and exclusion criteria

All patients coming to BPKIHS with a history of fever for 14 days or more and clinical splenomegaly were eligible for the study and were included after informed consent was given by the patient or his/her closest relative (for unconscious or paediatric patients). Only clinically suspect patients were thus included in the study. Indeed, as emphasized by Sackett *et al.* (1991), we considered it essential to validate both diagnostic tests on a patient group representative for those persons on whom physicians will use the tests in the future. Patients with prior treatment for kala-azar were excluded from the study. Serological diagnosis is known to be unreliable in such patients because of the long persistence of antibodies against *Leishmania donovani* after treatment.

Diagnostic procedures

All patients included were admitted to the medical ward for the initial diagnostic work-up and treatment. On day 0 (admission day), blood was drawn for complete blood count, chemistry, coagulation profile, thick and thin smear, blood cultures and HIV testing after pre-test counselling (Vironostica® and Recombigen® ELISA tests). Chest X-ray, abdominal ultrasound and other tests were performed at the physician's discretion.

rK39 dipstick test

The rK39-antigen-based dipstick test (InSure Rapid Test for Visceral Leishmaniasis® from InBios International, Seattle, USA) was performed using patient serum on day 0 or 1 by the same house officer throughout the study and the results were kept blinded to the physician in charge of the patient. The procedure was as follows: after allowing the serum specimen and the dipstick to reach room temperature, 20 µl of serum were added on the dipstick, which was then placed vertically in a test tube. Two drops of the chase buffer solution provided with the dipstick kit were added in the test tube. The results were read after 5 min and, if still negative, after 10 min. Even a weak band in the test region was considered as a positive result. The test was repeated if the control line remained negative after 10 min.

Direct agglutination test

Patients sera were kept frozen at −70 °C and the DAT was performed at BPKIHS every 3–6 months by a laboratory technician (M.L.D.) previously trained on-site by the chief laboratory technician of the Protozoology Unit of the Prince Leopold Institute of Tropical Medicine in Antwerp (ITMA). Results of the DAT were thus not used for clinical decisions. Crosschecking of the DAT was performed at ITMA from blood impregnated on filter papers on day 0 and stored in sealed plastic bags. The DAT antigen was prepared at ITMA using a modification of the method of El Harith *et al.* (1986) and described by Boelaert *et al.* (1999c). The liquid antigen was kept at 4 °C during transport and storage at BPKIHS. The test was carried out

in microtitre plates (V-shaped wells) with the necessary positive and negative controls. The test was read visually against a white background and the end-point titre was taken as the last well where agglutination was seen. A first serum dilution was tested at 1:400; when positive, full titration was performed (1:400 to 1:204 800).

Parasitological diagnosis

All patients underwent bone marrow aspiration on day 0 or 1. Careful microscopic search for the amastigote form of *L. donovani* (LD bodies) was carried out independently at both the Department of Microbiology and the Department of Pathology of BPKIHS by two examiners. When results were discordant, both examiners met in order to reach a consensus. Moreover, slides from discordant results were reviewed at the Parasitology Laboratory of the University Medical Centre of Montpellier (Dr J. Dereure). If bone marrow aspiration was negative for LD bodies, spleen aspiration was performed unless one or more of the following condition was present: coagulation disorder or platelet count $\leq 50\,000$ mm^{-3}, refusal of the patient or her/his physician, alternative diagnosis ascertained by initial work-up with a clear clinical response to specific therapy or spleen size ≤ 3 cm under the left costal margin.

Case definitions

A confirmed case of kala-azar was defined as a patient with positive parasitology on either bone marrow or spleen aspiration smear. A control was defined as an eligible patient in whom kala-azar was excluded by a negative bone marrow aspiration and by a subsequent negative spleen aspiration. Also, we considered as a control a patient with a negative bone marrow smear in whom a firm alternative diagnosis was reached. In addition, all control patients were carefully followed-up for 6 months. Patients showing any clinical or parasitological evidence of kala-azar during this 6-month follow-up have been excluded from our control group.

Case management

All patients were hospitalized until clinical improvement. Kala-azar patients with no prior history of complete anti-leishmanial treatment were treated with generic sodium stibogluconate (SAG from Albert David Ltd – Calcutta) 20 mg/kg/day for 30 days.

Statistical analysis

Data were entered at BPKIHS in an Excel data sheet and electronically sent on a weekly basis to the principal investigator. Kala-azar and control patients were compared on socio-demographic, clinical and laboratory characteristics, using cross-tabulations and chi-square tests for categorical variables and comparison of means and *t*-test for continuous variables. Sensitivity, specificity, positive and negative predictive values were calculated for the rK39-antigen-based dipstick test and for each dilution of the DAT, as well as their 95% confidence intervals. The performance of the two DATs (BPKIHS and ITMA) was analysed by comparing the area under the curve (AUC) of the corresponding receiver-operating characteristics curves with a statistical software (Statistical Package for Social Sciences, v. 10.1.0). All statistical tests were two-tailed, with a significance level of 0.05.

Results

A total of 227 clinically suspect patients were admitted to BPKIHS during the 14-month study period. Of these, 195 patients were included in the study after exclusion of three patients because of early defaulting and 29 patients (25 kala-azar cases, four controls) because of previous treatment for kala-azar. Seven patients were later excluded from analysis because their physician treated them for kala-azar despite the absence of parasitological evidence and four patients were excluded from the control group because kala-azar was diagnosed during follow-up.

The analysis of the validity of the rK39 dipstick and the DAT was performed in the remaining 184 patients: 108 males and 76 females with a mean age of 23 years. There were 139 kala-azar patients and 45 controls. The proportion of kala-azar among clinical suspects was 76%. The diagnosis of the 139 kala-azar patients was made by a positive bone marrow (131 patients) or spleen (eight patients) aspiration.

All 45 controls had a negative bone marrow aspiration but spleen aspiration was performed for only six patients. The main reasons for not performing spleen aspiration were a clear initial response to treatment of an alternative diagnosis (17 patients), a refusal of the patient or his physician (13 patients), a spleen too small to be punctured (eight patients) and a coagulation disorder (one patient). The discharge diagnosis of the 45 controls was malaria (20 patients), disseminated tuberculosis (five patients), enteric fever (four patients), leukaemia (three patients), other haematological disorders (three patients), infectious endocarditis (two patients), sepsis (two patients), solid malignancy (one patient) and other infections (five patients). Follow-up was completed at 1, 3 and 6 months for 29 of 45 controls (64%).

The presence of weight loss, skin darkening and the absence of cervical lymph nodes were significantly more

frequent in kala-azar patients and the mean platelet count was significantly lower in kala-azar patients (data not shown). HIV testing was negative for all 184 patients.

The InSure® dipstick was performed on the 184 patients. The result was positive in 148 patients and negative in 36 patients. The sensitivity of the InSure® dipstick is 97% (95% CI: 92–99%) and the specificity is 71% (95% CI: 56–81%). The positive and negative predictive values of the dipstick test are 91% (95% CI: 85–95%) and 89% (95% CI: 73–95%), respectively. The diagnosis of kala-azar would have been missed in four patients. Three out of the four patients had also a low DAT titre (≤1:800) but one patient had a DAT titre of 1:25 600. The dipstick was positive in 13 controls. Final diagnosis of these patients was malaria (10 patients), enteric fever (two patients) and disseminated TB (one patient). These patients were treated according to their clinical diagnosis and had a good initial response. Nine out of the 13 patients were followed-up at 6 months and were all considered as definitely cured.

The DAT was performed on 183 patients. One serum tube from a kala-azar patient was lost. The performance of the DAT depends on the cut-off titre chosen (Table 1). When compared with the DAT performed at BPKIHS, the DAT performed at ITMA shows similar sensitivity values but a trend towards higher specificity (data not shown). The AUC of the DAT performed at BPKIHS is 0.858, as compared to 0.890 for the DAT performed at ITMA (Figure 1). This difference is not significant ($P = 0.25$).

The predictive values calculated for both the rK39 dipstick and the DAT would vary greatly according to the proportion of kala-azar in clinical suspect patients (Figure 2). For a prevalence of 50%, for example, the InSure® dipstick would have a positive predictive value (PPV) of 77% and a negative predictive value (NPV) of 96%, the DAT 1:400 a PPV of 60% and a NPV of 96%, the DAT 1:6400 a PPV of 74% and a NPV of 88% and the DAT 1:51 200 a PPV of 82% and a NPV of 81%.

The four patients initially classified as controls but later excluded because they developed kala-azar during follow-up interestingly had all a positive dipstick and, for three of them, a DAT > 1:6400 between 3 and 6 months before kala-azar was diagnosed.

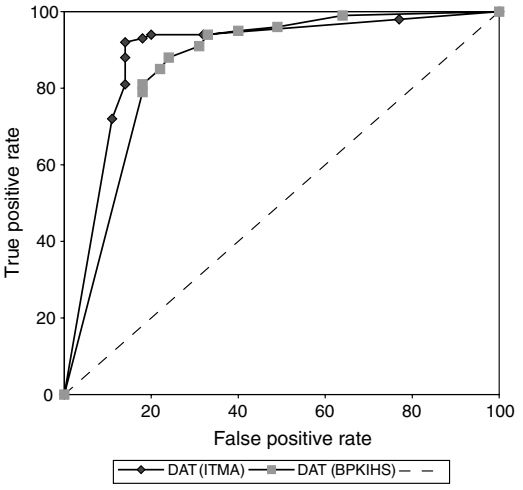

Figure 1 Receiver-operating characteristics curves of the DAT performed at ITMA and BPKIHS.

Table 1 Sensitivity, specificity and predictive values of the DAT performed at BPKIHS*

DAT cut-off titre	Kala-azar, no. of cases†	Controls, no. of cases	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
<1:400	2	16				
1:400	4	7	99 (94–99)	36 (22–49)	82 (76–87)	89 (64–96)
1:800	1	4	96 (90–98)	51 (36–64)	86 (79–90)	79 (60–89)
1:1600	1	3	95 (89–97)	60 (44–72)	88 (81–92)	79 (62–89)
1:3200	5	1	94 (89–97)	67 (51–78)	90 (83–93)	79 (62–88)
1:6400	4	3	91 (84–94)	69 (53–80)	90 (83–94)	70 (55–81)
1:12 800	4	1	88 (81–92)	76 (60–85)	92 (85–95)	67 (52–77)
1:25 600	5	2	85 (77–90)	78 (63–87)	92 (86–95)	63 (49–73)
1:51 200	3	0	81 (73–87)	82 (67–90)	93 (87–96)	59 (46–69)
1:102 400	109	8	79 (71–85)	82 (67–90)	93 (87–96)	56 (43–67)

* Values in parentheses are 95% confidence limits.
† One serum sample was lost.

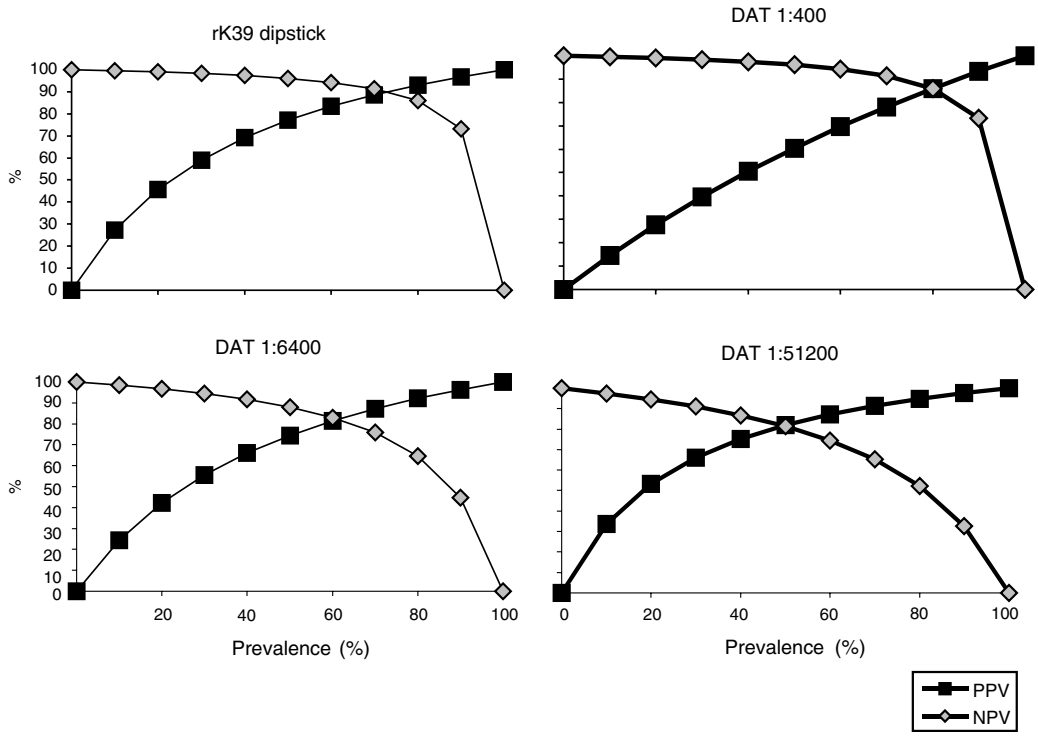

Figure 2 Positive (PPV) and negative (NPV) predictive values of the rK39 dipstick and the DAT with cut-off titres of 1:400, 1:6400 and 1:51 200 depending on the prevalence of kala-azar among clinical suspect patients.

Discussion

This validation study, conducted in a tertiary hospital of South-East Nepal where kala-azar is endemic, evaluated and compared the performance of the DAT and the rK39-based InSure® dipstick test for the diagnosis of VL among a group of 184 clinical suspect patients.

When interpreted with a cut-off titre of 1:6400, corresponding to the titre of 1:3200 found in the original report of El Harith *et al.* (1986), the DAT was 91% sensitive and 69% specific. When setting a higher cut-off titre (1:51 200), DAT specificity would increase only moderately to 82% but at the cost of lower sensitivity (81%). There was a non-significant trend towards a better specificity when the test was performed in the reference laboratory. The relative lack of reproducibility of the DAT, previously described by Boelaert *et al.* (1999c), did not significantly alter the results in our study considering the good conditions of transport and storage of the liquid antigen and the specific training received on site by the laboratory technicians.

The sensitivity of the DAT is invariably recorded above 90% in the literature when low or intermediate cut-off titres are used. However, its specificity varies greatly and depends to a large extent on the choice of the control group. DAT specificity is reported well above 90% when control groups are composed of non-symptomatic people or patients with known other diseases (except African trypanosomiasis), coming either from kala-azar endemic or non-endemic areas (El Harith *et al.* 1987; Sinha & Sehgal 1994; Schaefer *et al.* 1995; Boelaert *et al.* 1999a). When only clinically suspect patients are studied, a much more realistic situation for a physician, the reported DAT specificity falls to 58% (Boelaert *et al.* 1999b) and 72% (Zijlstra *et al.* 1991). Our findings of a specificity of 69% for a cut-off titre set at 1:6400 are consistent with these previous reports.

The DAT is a well-validated test for the diagnosis of kala-azar and has been widely used for more than 15 years. Because of the quantitative results obtained by gradual dilution, the cut-off titres to use can be (and should be) tailored for each epidemiological situation. The main limitation of the DAT is its relatively sophisticated procedure, which impairs its wide application in peripheral health structures and the fragile nature of the antigen in its liquid form, which is a source of logistic problem for transport and the likely cause of lack of reproducibility. The latter problems could be solved in the future by the use of freeze-dried antigen (Meredith *et al.* 1995; Zijlstra *et al.* 1997; Oskam *et al.* 1999). The relative high price of the DAT liquid antigen (4.5 US$ per test) is also a constraint but Boelaert *et al.* (1999d) showed that currently the cost of test-treatment strategies depend mainly on the cost of hospitalization and treatment.

The rK39-antigen-based InSure® dipstick showed a sensitivity of 97% and a specificity of 71% in our study. It proved to be very easy to perform with minimal training. Reproducibility of the dipstick was not assessed here but is clearly an issue to be considered in future studies. Indeed, the test line can be sometimes very faint and the distinction from the white background of the dipstick can be difficult. It is difficult to compare these results with those obtained from previous studies assessing the value of serology against rK39 antigen. The initial studies showed excellent sensitivity and specificity but used an ELISA format that is not practical to use in most health facilities in kala-azar endemic areas (Badaro *et al.* 1996; Zijlstra *et al.* 1998). A well-designed study from Sundar *et al.* (1998) showed excellent results (sensitivity: 100%; specificity: 98%) of an rK39-antigen-based test on a dipstick format in India but these results were challenged by a low sensitivity (67%) found by Zijlstra *et al.* (2001) in Sudan. The manufacturer of the dipstick tested in the two latter studies has now abandoned its production. A second generation of InSure® dipstick is currently produced and commercialized and is designed to be more specific than the first-generation dipstick tested in our study. A preliminary retrospective study in Nepal showed a sensitivity and specificity of 100% of this second-generation dipstick but only 14 VL patients were included and the control group was composed of non-symptomatic villagers with no personal or household history of VL (Bern *et al.* 2000).

The transition of rK39-antigen-based serological test from its very accurate ELISA format to a very practical to use dipstick format is not without difficulties. With the current format expressing only a qualitative result (positive or negative), it will be difficult for manufacturers to produce a dipstick with both high sensitivity and specificity applicable in all kala-azar endemic areas. An alternative approach would be to produce a dipstick with two test lines (or two separate dipsticks): one detecting low antibody titre for screening and one detecting high antibody titre for potentially confirming the diagnosis. The dipstick tested in this study has a high potential for use as a screening test for kala-azar in peripheral health structures where parasitological diagnosis and the DAT cannot be used for technical reasons. This will apply to the currently produced second-generation InSure® dipstick only if its sensitivity remains unchanged. Another constraint for the InSure® dipstick is the sustainability of production by a private company of a diagnostic test for kala-azar, a neglected disease with low profit potential.

The specificity of the InSure® dipstick and the DAT might have been underestimated in our study because of the imperfection of our gold standard. Indeed, only six of the 49 patients with an initial negative bone marrow underwent spleen aspiration. Because of the limited sensitivity of bone marrow aspiration, some diagnosis of kala-azar might have been missed. We believe that the number of missed cases is small considering that only four of the 49 (8.2%) initial controls were diagnosed with kala-azar during follow-up and that 6-month follow-up was completed in two-thirds of the patients. Boelaert *et al.* (1999b) showed in a group of 149 clinical suspect patients that specificity of the DAT raised from 68% to 85% when a mathematical model (Latent Class Analysis) was applied to correct for the imperfection of the parasitological gold standard (which did not include spleen aspiration). Furthermore, the confidence intervals of the specificity values are relatively wide in our study because of the limited number of patients included in the control group.

The specificity of serological tests for VL is anyway affected by the long persistence of antibodies after *L. donovani* infections, which are most frequently sub-clinical. Hailu (1990) showed in Ethiopia that the DAT remained positive in most kala-azar patients up to 7 years after treatment. Antibodies targeted against the more specific rK39 antigen have been shown to be detected by a strip test significantly less frequently than antibodies detected by the DAT 12 months after completion of treatment of kala-azar patients in Sudan (Zijlstra *et al.* 2001). However, the strip test used in this study was lacking sensitivity and IgG directed against rK39 have been detected up to 24 months post-treatment in Sudan by the more sensitive ELISA technique (Zijlstra *et al.* 1998). Because of this long persistence of antibodies, clinical suspect patients with a positive rK39 dipstick or an elevated DAT titre who have a prior history of kala-azar should have a confirmatory bone marrow or spleen aspiration performed.

Despite the relative lack of specificity of both the InSure® dipstick and the DAT, both tests can be used as fairly reliable confirmatory tests at BPKIHS with positive predictive values of, respectively, 91% and 90% (cut-off titre: 1:6400). This would not apply in areas where the proportion of kala-azar among clinically suspect patients is lower. In these areas, spleen puncture could be used for confirmation of the diagnosis or decision analysis strategies aiming at increasing the pre-test probability of kala-azar before initiating treatment could be evaluated and applied.

Both the InSure® dipstick and the DAT with a very low cut-off titre (1:400) have a negative predictive value of 89% and can be used at BPKIHS as fairly reliable screening tests. The proportion of kala-azar among clinical suspects is high at BPKIHS (76%) because of its status as a reference tertiary hospital and its situation in a low endemic area for malaria, a usual alternative diagnosis. Both tests are very sensitive and would perform much better as screening tests in areas where the proportion of kala-azar within patients tested is 50% or lower, a situation found in East Africa, for example, where malaria is much more frequent. For a proportion of 50%, for example, the negative predictive value of the dipstick and the DAT (cut-off titre 1:400) would be 96%.

None of the 184 patients included in this study were infected with HIV. The sensitivity of several serological tests being decreased in HIV–kala-azar co-infected patients (Rosenthal *et al.* 1995; Medrano *et al.* 1998), the performance of the DAT and the Insure® dipstick shown here should not be extrapolated to settings where HIV–kala-azar co-infection rate is high.

In summary, our results show that in Nepal, the potential of the first-generation Insure® dipstick test to be used as a screening test for VL in peripheral health structures is excellent. Patients with a positive dipstick could then be referred to secondary or tertiary hospitals for diagnosis confirmation (by bone marrow or spleen aspiration or by the DAT with a high cut-off titre) and for specific treatment.

Before definite guidelines of utilization of these tests in Nepal can be drawn, the performance of the currently produced second-generation Insure® dipstick needs to be prospectively assessed in clinical suspect patients. Such evaluation has just been completed in Nepal and results should be published soon. Moreover, the feasibility, reproducibility and predictive values of these tests, when performed in more peripheral health structures, are being assessed in a district hospital based study in the same region.

The ongoing fight led by numerous medical organizations for a better access in developing countries to essential drugs for diseases like AIDS, multidrug resistant tuberculosis, African trypanosomiasis or leishmaniasis should include the support of development and production of standardized, affordable, practical and reliable diagnostic tests. Both the lack of diagnosis of kala-azar, a deadly disease if left untreated, and the overdiagnosis of this condition, exposing patients to relatively toxic drugs, can have fatal consequences for the patients.

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A COMPARATIVE STUDY OF THE EFFECTIVENESS OF DIAGNOSTIC TESTS FOR VISCERAL LEISHMANIASIS

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Abstract. We compared the validity of pancytopenia, the formol-gel test (FGT), the indirect fluorescence antibody test (IFAT), the direct agglutination test (DAT), and the rK39 dipstick test as diagnostic criteria for visceral leishmaniasis (VL) in Nepal. Between September 2000 and January 2002, 310 clinical suspects had a bone marrow aspirate, and if negative, a spleen aspirate smear examined for *Leishmania donovani*. Sensitivity and specificity of all tests were determined compared with parasitology and by latent class analysis (LCA). Compared with parasitology, the sensitivities of the other tests were as follows: pancytopenia = 16.3% (95% confidence interval [CI] = 11.3–22.5%), FGT = 39.9% (95% CI = 32.7–47.4%), IFAT = 28.4% (95% CI = 22.0–35.5%), DAT = 95.1% (95% CI = 90.8–97.7%), and the rK39 dipstick test = 87.4% (95% CI = 81.7–91.9%). Sensitivity estimates obtained by LCA were similar, but specificity estimates were substantially higher (DAT = 93.7% versus 77.8%; rK39 dipstick test = 93.1% versus 77.0%). The DAT or the rK39 dipstick test can replace parasitology as the basis of a decision to treat VL in Nepalese peripheral health services.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is considered as one of the most neglected tropical diseases.[1] Communities affected by kala-azar often live in remote areas and have poor access to health services. The need for innovation in VL chemotherapy and diagnosis was recently highlighted.[2] Treating patients on clinical presumption is inadequate because of the potentially serious side effects of current chemotherapy. Direct microscopic examination and/or culture of spleen tissue aspirate is the reference test for diagnostic confirmation, but this technique is hardly recommendable in the first-line health services in endemic areas. Alternatively, bone marrow and lymph node aspirates are used, but these methods are substantially less sensitive.[3] Past research aimed at developing a cheap and reliable serologic test that could replace parasitology. El Harith and others developed a direct agglutination test (DAT) in the 1980s and proposed it as a test for field use.[4] However, some investigators disagreed because of a presumably low specificity in the clinical setting.[5] The effectiveness of the DAT proved satisfactory in a large multi-center study.[6] More recently, high validity was reported for the rK39 dipstick test,[7] but this was contested soon after the initial report.[8,9] Moreover, commercial production of the dipstick test was interrupted several times.[9]

Diagnostic research in VL has been hampered by the lack of a gold standard. As Staquet and others[10] showed, a flawed reference test can substantially affect validity estimates of new tests under scrutiny. Thus, the published sensitivity and specificity estimates of serologic tests might be biased to some extent, depending on the controls and reference test used. Parasitology for VL is very specific, but unless spleen aspirates can be taken, its sensitivity is less than 90%.[3] The allegedly low specificity of serologic tests for VL, along with problems of regular supply, have so far prevented their introduction into routine health service practice. For example, in rural Nepal, most VL patients in peripheral health facilities are still treated on the basis of clinical suspicion and/or the result of a formol-gel test (FGT).[11]

To avoid biased estimates and needless discussions, a better and more standardized validation methodology is needed. Hadgu and Qu[12] suggested latent class analysis (LCA) could be a potential solution to the gold standard problem. This method of analysis is a mathematical technique that models associations between observed variables that imperfectly measure a non-observable (latent) variable.[13,14] When a diagnostic test is validated in a group of people, their true disease status can be considered as a latent variable with two mutually exclusive and exhaustive classes or categories: diseased and non-diseased. The LCA model estimates disease prevalence and sensitivity and specificity of all the diagnostic tests. So far, LCA has been used extensively in psychiatric research and in the social sciences, but experience in biomedical research is still limited. To clarify the current options in VL diagnostics in endemic areas, we validated three existing (FGT, DAT and an indirect fluorescence antibody test [IFAT]) and one novel serologic test (rK39), as well as a hematologic sign (pancytopenia) in a clinical care setting. We used classic contingency table analysis as well as LCA to corroborate the findings.

METHODS

Patients. The data were collected at the B.P. Koirala Institute of Health Sciences (BPKIHS) in Dharan in the Morang District of Nepal. This is a 648-bed tertiary care center serving the eastern region of Nepal, which includes VL-endemic areas. Patient recruitment aimed at enrolling at least 100 true cases of VL and at least 100 true non-cases to achieve adequate precision for the sensitivity and specificity estimates of the tests. Therefore, the total sample size to enroll was fixed at 300 clinical suspects given the expected prior probability of 70% VL cases in a group of presenting clinical VL suspects.[9] Patients were enrolled consecutively until the required sample size was achieved.

Clinical suspicion for VL was defined as a history of fever of 14 days or more and splenomegaly. Children of very young age were excluded. All clinically suspected adult and pediatric patients who presented to the Out-Patient Department and Emergency rooms between September 2000 and January 2002 were enrolled on the condition that they (or their guardian) gave informed consent.

A questionnaire with clinical and epidemiologic data was filled out for each patient at enrollment and diagnostic procedures were performed: bone marrow aspiration and the leishmanin skin test. Also, a venous blood sample and a urine sample was taken. If the bone marrow aspirate was negative, and malaria had been ruled out, a spleen aspirate was proposed. All VL patients were offered free treatment in accordance with current policy at BPKIHS and World Health Organization guidelines. The Institutional Review Board of the BPKIHS and of the Prince Leopold Institute of Medicine in Antwerp (ITMA) gave ethical clearance for the study.

Diagnostic criteria and tests. *Pancytopenia.* Pancytopenia was defined as a hemoglobin level < 11 g/dL, a white blood cell count < 4×10^9/L, and a platelet count < 100×10^9/L.

Formol-gel test. Twenty microliters of 40% formaldehyde (Glaxo India, Ltd., Bombay, India) was added to 200 μL of serum in a glass tube, and after 20 minutes the gelification reaction was visually assessed as positive or negative.

Indirect fluorescence antibody test. The IFAT was carried out at the Unidade de Leishmanioses of the Institute of Hygiene and Tropical Medicine in Lisbon, Portugal according to the procedure of Lanotte[15] using cultured promastigotes of *Leishmania (Leishmania) infantum* MON-1 as antigen. Washed promastigotes were dispensed on immunofluorescent microscope slides, air-dried and kept at -70°C until use. Total anti-human immunoglobulins conjugated with fluorescein isothiocyanate and diluted 1:100 (BioMérieux, Lyon, France) was used. A cut-off value ≥ 1:32 was used to establish a positive IFAT result for a child < 15 years old and a value ≥ 1:128 was used for adults.

Direct agglutination test. The DAT was performed at BPKIHS on 1 μL of serum according to standard procedures.[4,6] The antigen was produced at ITMA as follows. Promastigotes of the reference batch *L. (L.) donovani* 1-S were cultivated in a temperature-controlled cell shaker in glucose lactalbumin serum hemoglobin.[16] Log phase promastigotes were harvested and treated with 0.4% trypsin, fixed with 4% formaldehyde, stained with 0.025% Coomassie blue, and stored at 4°C until use. This DAT antigen was mixed in V-shaped well microtiter plates (Greiner-Bio One GmbH, Frickenhausen, Germany) with patient serum dilutions between 1:200 and 1:204,800, and after 12 hours the agglutination reaction was visually assessed against a white background. The end titer was read as the dilution immediately before the well with a clear sharp-edged blue spot identical in size to the negative control. For the analysis, a DAT titer > 1:3,200 was considered positive.

rK39 dipstick test. The rK39 dipstick test (lot numbers AF1008 and BF1019, InSure Rapid Test for Visceral Leishmaniasis®; InBios International, Seattle, WA) was performed at BPKIHS.[17] At room temperature, 30 μL of serum was added to the dipstick, which was then placed vertically in a test tube. Two drops of the chase buffer solution provided with the dipstick kit were added to the test tube. The results were read after five minutes and, if still negative, after 10 minutes. Even a weak band in the test region was considered a positive result. The test was repeated if the control line remained negative after 10 minutes.

Parasitology. Two independent readers at BPKIHS performed direct semi-quantified microscopic examination of methanol-fixed (10 minutes), Jenner-stained (five minutes), Giemsa-stained (15 minutes) smears of bone marrow or spleen aspirate. Two independent readers at ITMA performed quality control on 10% of the slides.

Statistical analysis. The data were processed with SPSS for Windows version 10.0.5 (SPSS, Inc., Chicago, IL), as well as with Latent Gold® version 2.0.18 (Statistical Innovations, Inc., Belmont, MA). Sensitivity and specificity were estimated by the classic validation method as well as by LCA modeling. We used parasitology as the reference test in the 2 × 2 contingency table: a patient with a positive bone marrow aspirate and/or spleen aspirate was considered a kala-azar case. In LCA, we started by fitting the basic two latent class model. Given a group of individuals with unknown disease status, for whom results from several diagnostic tests are available, LCA will model the probability of each combination of test results conditional on latent class, i.e., disease status. In basic latent class models, tests are taken to be conditionally independent (conditional on latent class), i.e., there are no associations between test errors within each category of the latent variable (diseased/not-diseased). Subsequently, more advanced models were fitted in which this condition was relaxed.[12,18] Model fit was assessed on the basis of the Bayes information criterion.[19] We report sensitivity and specificity of tests with an approximate 95% confidence interval (1.96 × SE) as estimated by the best model.

RESULTS

Between September 6, 2000 and December 23, 2001, 310 patients with febrile splenomegaly syndrome were enrolled in the study. Bone marrow aspirates were negative for 132. However, spleen aspirates could not be obtained in 101 (76.5%) because of contraindications or patient refusal. A diagnosis of kala-azar was reached by parasitologic confirmation in 184 (59.4%) of the 310: 178 by positive bone marrow and six by spleen aspirate.

Three confirmed VL syndromes were re-admissions of relapse cases. The 181 confirmed new kala-azar patients had a median age of 25 years (25th percentile = 13 years, 75th percentile = 36 years). The male:female ratio was 1.7:1. The median duration of fever at first admission was eight weeks (25th percentile = 3.5 weeks, 75th percentile = 11 weeks) and mean ± SD spleen size was 6.1 ± 3.7 cm below the costal margin. Anemia was present in 166 (91.7%) of 181, leukopenia in 104 (57.5%), and thrombocytopenia in 41 (22.7%). Pancytopenia was present in 29 (16%) of the 181 patients.

There were 126 febrile splenomegaly syndromes with a negative parasitology. For 13 (10.3%) of them, the clinicians indicated that kala-azar was the most probable diagnosis on the basis of the overall case history and clinical picture. In 91 (72.2%), an anatomopathologically or microbiologically confirmed alternative diagnosis was reached. For 20 (15.9%) patients, such an alternative diagnosis was deemed most likely on clinical grounds. Two patients left against medical advice before a final clinical diagnosis was reached.

The validity of pancytopenia and serologic tests as diagnostic markers for VL in the 310 febrile splenomegaly syndromes is shown in Table 1. When compared with the results of parasitology as the reference test, pancytopenia, the FGT, and the IFAT had low sensitivity, but very high specificity. The DAT and rK39 dipstick test had high sensitivity, but moderate specificity. The quality control done on the parasitology smears showed perfect concordance for grade 2+ or higher, but for smears with scanty parasites (grade 1), there was considerable discrepancy.

The validity of pancytopenia and diagnostic tests (including parasitology) as estimated by the best LCA model is shown in Table 1. This best model was reached in a modeling strategy comparing nine models (Table 2). A basic two latent class model did not fit the data very well because there was considerable residual correlation between the FGT and IFAT, between the IFAT and pancytopenia, and between the FGT and parasitology. Therefore, we fitted several other two latent class models including direct effects between pairs of tests.[18] Model 5, which included a direct effect between the FGT and IFAT, gave the best fit. The expected frequencies of diagnostic test patterns, as well as the probability of belonging to latent class VL predicted by this model, are shown in Table 3.

The LCA estimates for sensitivity of pancytopenia, the FGT, and IFAT, as estimated by the best-fitting LCA model, were low, whereas the DAT showed high sensitivity and the rK39 dipstick test and parasitology showed good sensitivity (Table 1). Almost exactly the same sensitivity estimates were obtained by both validation methods. The specificity of DAT and the rK39 dipstick test was good and substantially higher in LCA than when estimated by classic validation. The discriminatory power of each test based on LCA results is shown in Figure 1. The estimated prevalence of VL in this group of patients was 65.4% (n = 309) in LCA versus 59.2% (n = 310) according to parasitologic results only and 64.0% (n = 308) according to the clinicians' final diagnosis.

DISCUSSION

The main finding in our study was that the sensitivity and specificity of the DAT and rK39 dipstick test compared favorably in the diagnosis of VL with that of parasitologic examination of bone marrow combined with spleen aspirates. Moreover, our study illustrates the problems related to parasitology as a reference test for the validation of VL diagnostics.

To our knowledge, no study so far has addressed the performance of VL diagnostics by a comparative assessment on the same patients from an endemic area. Although our patients were certainly representative for the clinical suspects presenting to referral hospitals in eastern Nepal, this does not necessarily imply that frequency and stage of VL would be the same in patients presenting to first-line health services. Serologic test performance is known to be dependent on stage of the disease, so one must be careful in extrapolating results. The rK39 dipstick test format evaluated in this study was a prototype version distributed in 2000 by the company for research purposes only. Although similar to the currently commercialized version (Raychaudhury S, 2003, personal communication), discrepancies between our findings and those of other investigators[9] might be explained by the fact that they evaluated earlier prototypes.

Validation studies of VL diagnostics are usually based on parasitologic examination as a reference test. This should not be too problematic if spleen aspirates can be used for case ascertainment in all subjects, but this is rarely the case. In this tertiary care setting, it was not possible to obtain spleen aspirates for all patients with a negative bone marrow because of contraindications or patient refusal. When a reference test with sub-optimal sensitivity for case ascertainment is used, true VL cases are missed and therefore included in the group of controls. They will generate a positive result in any new test one wishes to evaluate (assuming this new test is 100% sensitive). For those cases, the new test is actually right while the reference test is wrong, and the specificity of the new test will thus be systematically underestimated. To prevent this kind of bias, our study included a multivariate analysis of the data by LCA, a technique based on loglinear modeling. LCA confirmed that parasitology, even though optimized by the combined use of bone marrow and spleen aspirates, had a lack of sensitivity as a reference test in this setting. As anticipated, the specificity estimates of the DAT and rK39 dipstick test were considerably higher in LCA than in classic validation. The specificity estimates of the IFAT and FGT were less affected because these tests were also less sensitive. Remarkably, LCA did not estimate the specificity of parasitology as 100% as one would expect on theoretical grounds. The reading of smears for *L. donovani* bodies is not easy. Our quality control showed discrepancies in the low-graded smears (grade 1), and some smears might actually have been wrongly taken as positive. This demonstrates again that parasitology cannot

TABLE 1

Validity of pancytopenia and diagnostic tests compared to parasitology, and as estimated by the best model in latent class analysis (CA)*

	Compared to parasitology†		LCA‡ (n = 309)§	
	Sensitivity % (n = 183)§	Specificity % (n = 126)	Sensitivity %	Specificity %
Pancytopenia	16.3 (11.3–22.5)¶	96.8 (92.1–99.1)	16.0 (10.9–21.1)	98.4 (95.8–100.0)
FGT	39.9 (32.7–47.4)	95.2 (89.9–98.2)	33.7 (26.5–40.9)	98.5 (95.8–100.0)
IFAT	28.4 (22.0–35.5)	94.4 (88.9–97.7)	30.0 (22.9–37.0)	98.3 (92.0–100.0)
DAT	95.1 (90.9–97.7)	77.8 (69.5–84.7)	96.9 (94.1–99.8)	93.7 (88.0–99.4)
RK39 dipstick test	87.4 (81.7–91.9)	77.0 (68.6–84.0)	90.1 (85.7–94.6)	93.1 (87.5–98.6)
Parasitology	Reference	Reference	88.1 (83.2–92.9)	94.8 (89.9–99.7)

* FGT = formol-gel test; IFAT = indirect fluorescence antibody test; DAT = direct agglutination test.
† Estimate with 95% exact binomial confidence interval.
‡ Estimate with approximate 95% confidence interval (1.96 × SE).
§ One serum sample was missing in the group with positive parasitology.
¶ n = 184.

TABLE 2
List of models fitted to the data*

Models	Number of latent classes†	LL	BIC	df	Model *P*
1 A, B, C, D, E, F	1	607.918	281.118	57	3.60×10^{-93}
2 X, A|X, B|X, C|X, D|X, E|X, F|X	2	85.675	–200.992	50	0.0013
3 X_3, A|X_3, B|X_3, C|X_3, D|X_3, E|X_3, F|X_3	3	44.6516	–201.882	43	0.4
4 X_4, A|X_4, B|X_4, C|X_4, D|X_4, E|X_4, F|X_4	4	30.6552	–175.745	36	0.72
5 X, A|X, BC|X, D|X, E|X, F|X	2	55.6076	–225.326	49	0.24
6 X, AC|X, BC|X, D|X, E|X, F|X	2	50.3012	–224.899	48	0.38
7 X, AC|X, BC|X, BF|X, D|X, E|X	2	46.0745	–223.393	47	0.51
8 X, AC|X, BC|X, BF|X, CE|X, D|X	2	39.7886	–223.945	46	0.73
9 X, AC|X, BC|X, BE|X, BF|X, CE|X, D|X	2	37.3878	–220.613	45	0.78

* LL = loglikelihood; BIC = Bayes information criterion; df = degrees of freedom; A = pancytopenia; B = FGT; C = IFAT; D = DAT; E = rk39 dipstick test; F = parasitology; X = latent variable (disease status); A|X = probability of test result A depends on disease status; BC|X = probability of test results B and C depends on disease status but also on direct effect between B and C. For definitions of other abbreviations, see Table 1.

claim the gold standard role, and the usefulness of complementary approaches such as LCA for validation.

Which of the five diagnostic markers we evaluated could then play a role in the first-line health services in this region of Nepal? Clinicians in tertiary hospitals rely on pancytopenia as a sign for VL when present in a case of febrile splenomegaly. Our data showed its specificity was extremely high, but unfortunately, its sensitivity was not. Furthermore, since laboratory infrastructure for hematology is limited in peripheral health services, this criterion might not be very helpful in the field. Interestingly, the specificity of the FGT was high in this study, but its sensitivity was low. The FGT is known to show positive results quite late in the disease process, and requires at least three months evolution, which may explain part of the low sensitivity in this study.

The performance of a MON-1/*L.infantum*-based IFAT using the same diagnostic cut-off titer as the reference values of the Portuguese laboratory showed low sensitivity in this area endemic for *L. donovani*, an observation that was already made in Sudan.[20] However, a receiver-operator characteristic analysis of the IFAT at several age-independent cut-off values showed good discrimination. An age-independent IFAT cut-off value for positivity $\geq$ 1:16 had a sensitivity of 87.4% (95% confidence interval [CI] = 81.7–91.9%) and a specificity of 77.0% (95% CI = 68.6–84.0%). Other investigators have reported comparable sensitivity but higher specificity of

TABLE 3
Observed and expected frequencies of tests patterns and probability of visceral leishmaniasis (VL) as estimated by latent class analysis model 5*

Pancytopenia	FGT	IFAT	DAT	Rk39 dipstick	Parasitology	Observed frequency	Estimated frequency	Probability of VL as estimated by model 5
–	–	–	–	–	–	88	85.9766	0.0004
–	–	–	–	–	+	4	4.9455	0.0479
–	–	–	–	+	–	6	6.7107	0.0436
–	–	–	–	+	+	2	2.5157	0.8602
–	–	–	+	–	–	6	6.8152	0.1477
–	–	–	+	–	+	13	7.7639	0.959
–	–	–	+	+	–	14	9.628	0.9549
–	–	–	+	+	+	63	68.0188	0.9997
–	–	+	–	+	–	1	0.0974	0.5228
–	–	+	+	–	–	1	0.2173	0.8064
–	–	+	+	+	+	12	11.8322	1
–	+	–	–	–	–	1	0.921	0.0125
–	+	–	+	–	+	2	2.6889	0.9987
–	+	–	+	+	–	2	3.3207	0.9986
–	+	–	+	+	+	30	24.5246	1
–	+	+	–	–	+	1	0.0919	0.9763
–	+	+	–	+	–	1	0.1138	0.9739
–	+	+	+	+	–	2	3.4831	0.9999
–	+	+	+	+	+	26	25.7573	1
+	–	–	–	–	–	1	1.3781	0.0044
+	–	–	–	–	+	1	0.1204	0.3759
+	–	–	–	+	+	1	0.4191	0.9866
+	–	–	+	–	+	1	1.4278	0.9964
+	–	–	+	+	–	1	1.7637	0.9961
+	–	–	+	+	+	10	12.9926	1
+	–	+	+	+	–	2	0.3058	0.9998
+	–	+	+	+	+	3	2.2608	1
+	+	–	+	+	+	4	4.686	1
+	+	+	+	–	+	1	0.5389	1
+	+	+	+	+	+	9	4.9216	1

* For definitions of other abbreviations, see Table 1.

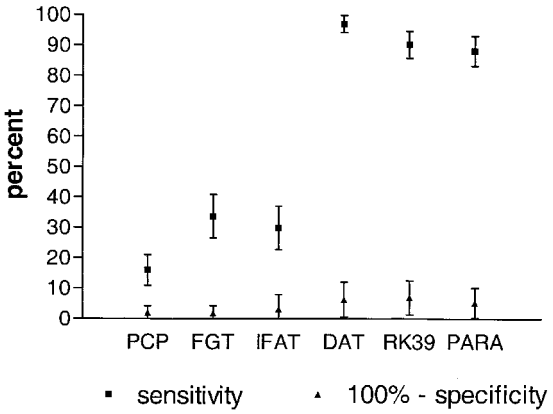

FIGURE 1. Sensitivity (%) and 100% - specificity of several diagnostic markers for visceral leishmaniasis. PCP = pancytopenia; FGT = formol-gel test; IFAT = indirect fluorescence antibody test; DAT = direct agglutination test; RK39 = rK39 dipstick test; PARA = parasitology. Bars show the 95% confidence intervals.

the IFAT when used in areas endemic for *L. infantum*.[21,22] Because the IFAT cannot be regarded as an option for peripheral health services in Nepal because of technologic constraints, we did not explore this further.

This study corroborated the known high sensitivity and the more than acceptable specificity of the DAT.[6] The features of the rK39 dipstick test format and of parasitology were found comparable to DAT in this study. In peripheral health services, the DAT or rK39 dipstick test could replace parasitology as the basis of a decision to treat, if one looks only at the validity criteria, while simplicity of use favors the dipstick format. However, deciding which test to include in diagnostic algorithms for VL should include other criteria, such as disease prevalence and health service context, as well as test reproducibility, cost, and sustainability.

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Evaluation of a urinary antigen-based latex agglutination test in the diagnosis of kala-azar in eastern Nepal

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Summary

BACKGROUND We evaluated the diagnostic accuracy as well as the reproducibility of the urine latex agglutination test 'KAtex' in the diagnosis of kala-azar in patients recruited at a tertiary care centre in Dharan, Nepal, between November 2000 and January 2002.

METHODS All patients presenting with fever of 2 weeks or more and splenomegaly were consecutively enrolled. Bone marrow and – if negative – spleen aspirates were examined for *Leishmania donovani*. Serum and urine samples were taken in duplicate for the Direct Agglutination Test (DAT) and KAtex. The reference laboratory determined sensitivity and specificity of KAtex. Reproducibility between both laboratories was assessed.

RESULTS KAtex was performed on urine from 155 parasitologically confirmed kala-azar and 77 non-kala-azar cases (parasitology and DAT-negative). KAtex showed a sensitivity of 47.7% (74/155, 95% CI: 39.7–55.9) and a specificity of 98.7% (76/77, 95% CI: 93.0–100.0). Reproducibility of KAtex showed a kappa of 0.684 ($P < 0.001$, $n = 232$).

CONCLUSION KAtex evaluation showed high specificity, low sensitivity and moderate reproducibility. A urine test for kala-azar could become a real breakthrough in kala-azar management if its reproducibility and sensitivity could be further improved.

keywords visceral leishmaniasis, sensitivity and specificity, diagnostic accuracy, urine antigen detection test, Nepal

Introduction

Visceral leishmaniasis (VL) or kala-azar is considered a major public health problem in Nepal where it is endemic in the south-eastern Terai region, with an estimated 5.5 million people at risk of acquiring the disease (HMGN 2001/2002). The recommended method for diagnosis of VL is the microscopic demonstration of amastigotes of *Leishmania donovani* (LD bodies) from spleen or bone marrow aspiration, although the sensitivity of the latter technique has been shown to be only 70–86% (WHO 1984; Zijlstra *et al.* 1992). Moreover, these techniques require expertise both from the physician and the laboratory technician and in Nepal LD microscopy is limited to reference hospitals. As the disease affects the poorest of the poor in remote rural regions, most patients lack access to these reference centres. The diagnosis of VL in those areas is thus usually based on non-specific clinical features (cachexia, anaemia, prolonged fever and hepato-splenomegaly) along with a positive formol-gel test.

Serological tests for the detection of antibodies have been developed in the pursuit of an alternative to parasitology. Their main advantage is non-invasiveness but they do not discriminate between clinical, subclinical or past infection, and cross-reaction with other pathogens is possible. The Direct Agglutination Test (DAT) developed by El Harith *et al.* (1986, 1988) has excellent diagnostic accuracy (Boelaert *et al.* 2004), but its use in Nepal has so far also been limited due to the expertise required for its execution. The indirect immunofluorescence antibody test (IFAT) requires an immunofluorescence microscope and is thus neither appropriate nor affordable for decentralized diagnosis. A test based on 39-amino acid repeat recombinant leishmanial antigen from *L. chagasi* (rK39) has been introduced in an enzyme-linked immunosorbent assay (ELISA) (Badaro *et al.* 1996; Zijlstra *et al.* 1998) and,

later, in a lateral flow dipstick format (Sundar *et al.* 1998). The latter is very easy to use in the field and the initial study showed 100% sensitivity and 98% specificity (Sundar *et al.* 1998). However, an evaluation in Sudan showed only 67% sensitivity (Zijlstra *et al.* 2001). Moreover, this particular format of the dipstick is no longer available. Another version tested in India proved to be a good diagnostic guide in kala-azar suspect cases (Sundar *et al.* 2002). Sarker *et al.* (2003) found in Bangladesh high sensitivity in confirmed kala-azar patients and high specificity in healthy endemic controls and patients suffering from other conditions. In Nepal, an early version of this dipstick showed a specificity of only 71% in controls who presented as clinical suspect cases of kala-azar (Chappuis *et al.* 2003); however, more encouraging results were obtained with later generations of this dipstick (Bern *et al.* 2000; Boelaert *et al.* 2004).

Recently, Sarkari *et al.* (2002) described a urinary leishmanial antigen. This is a low-molecular weight, heat stable carbohydrate detected in the urine of VL patients but not in the urine of patients suffering from malaria, schistosomiasis, or non-parasitic diseases including typhoid and brucellosis. An agglutination test for the detection of this urinary antigen has been evaluated in laboratory trials, using urine collected from well-defined cases and endemic and non-endemic controls. The test had 100% (95% CI: 98.8–100.0) specificity and sensitivity between 64 (95% CI: 42.5–82.0) and 100% (95% CI: 47.8–100.0) (Attar *et al.* 2001). In a field trial in Sudan the test was positive in all 15 microscopy-positive kala-azar cases [sensitivity 100% (95% CI: 78.2–95.2)] and was negative in 41 of 45 bone marrow and/or lymph node smear negative clinical suspect cases of kala-azar [specificity 87.2% (95% CI: 78.8–97.5)]*.

We evaluated the diagnostic accuracy and the reproducibility of the KAtex, a urinary leishmanial antigen-based latex agglutination test, in clinically suspect patients in an endemic kala-azar area in Nepal.

Materials and methods

Study population

The study was conducted at the B.P. Koirala Institute of Health Sciences (BPKIHS), a 648 bed University Hospital located in Dharan, in the south-eastern region of Nepal. BPKIHS serves as a reference tertiary level hospital for the eastern region, which includes several kala-azar endemic districts. The research protocol was approved by the ethical committee of BPKIHS in August 2000. Ethical clearance was also obtained from the institutional review board of the Institute of Tropical Medicine in Antwerp (ITMA), Belgium. The patients were prospectively recruited from the outpatient department and the emergency department of BPKIHS between November 2000 and January 2002. Every patient presenting with a history of fever for 2 weeks or more and clinical splenomegaly was considered as a clinical suspect case and was enrolled if he/she gave written informed consent. For paediatric patients, informed consent was sought from the guardian.

Reference standard

Splenic aspiration is considered close to gold standard for VL diagnosis, but as it is an invasive procedure, bone marrow-negative patients often reject it at BPKIHS. Therefore, we used a combination of parasitology and serology as the reference standard for the evaluation of the KAtex. We considered those with positive microscopy in bone marrow or spleen as confirmed kala-azar cases. A non-kala-azar case was somebody with negative parasitology and negative serology (i.e. DAT titre ≤ 3200).

Diagnostic procedures

All patients enrolled were admitted to the medical wards for the diagnostic work up and treatment. On day 0 (admission day), blood was drawn for complete blood count, chemistry, coagulation profile, thick and thin smear for malaria parasite, blood cultures and HIV testing after pre-test counselling. Chest X-ray, abdominal ultrasound and other tests were performed at the physician's discretion. Serum was collected for DAT. A urine specimen was collected in duplicate at day 0 and stored at −70 °C until analysis.

Parasitological diagnosis

All patients had a bone marrow aspiration performed at day 0 or 1 and a microscopic search for the amastigote form of *L. donovani* (LD bodies) was carried out by the Department of Microbiology of BPKIHS. Giemsa-stained smears were designated as positive if LD bodies were seen or negative if no LD bodies were seen in 1000 oil immersion fields. If bone marrow aspiration was negative for LD bodies, spleen aspiration was performed except in those with prolonged prothrombin time, decreased platelets below 50 000/mm^3 or spleen <2 cm palpable below the costal margins. A parasite density score was determined microscopically at magnification objective 100 × eye piece 10 in the Giemsa-stained smear by use of a scale ranging

* Confidence intervals were computed by authors on the basis of published figures.

from 0 (no parasites per 1000 oil immersion fields) to +6 (>100 parasites per field) using the method originally developed for splenic biopsies (WHO 1990) but which has been successfully applied to the quantification of bone marrow smears both in BPKIHS and ITMA.

Two independent readers at BPKIHS read the slides, and, in case of discrepancy, the reading of a third more senior reader was decisive. For quality control, 10% of the positive and 10% of the negative slides were cross-checked in the same way at the Protozoology Unit, ITMA.

Direct Agglutination Test

The DAT was performed by a laboratory technician at BPKIHS who had been previously trained by the chief laboratory technician of the Protozoology Unit of ITMA. The DAT antigen was prepared at ITMA using a modification of the method of El Harith *et al.* (1986) and described by Boelaert *et al.* (1999). The liquid antigen was kept at 4 °C during transport and storage at BPKIHS. The test was carried out on microtitre plates (V-shaped wells) with the necessary positive and negative controls. The test was read visually against a white background and the end titre was read as the dilution immediately before the well with a clear sharp-edged blue spot identical in size to the negative control. For the analysis, a DAT titre >1:3200 was taken as positive.

Urine latex agglutination test

Urine samples were taken on the day of admission and kept frozen until analysis. The KAtex urine latex agglutination test (Kalon Biological Ltd, Aldershot, UK) was performed by one technician at both BPKIHS and ITMA. Both technicians were blinded to the patient's diagnosis. The KAtex kit consists of test latex, a positive and a negative control and a reusable glass test slide with a black background.

As pretreatment, 1 ml of urine was transferred into the sample tube and placed on a boiling water bath for 5 min. This was to inactivate heat labile material capable of causing a false positive reaction. Meanwhile, all test reagents were brought to the ambient temperature. Fifty microlitres of the treated urine sample was placed to a reaction zone in the glass slide and a drop of test latex was added to it. The liquids were stirred to a completely homogenous mixture and rotated continuously for 2 min. For every assay, a negative control in the reaction zone next to the test sample was run. Any agglutination discerned when compared with the negative control was considered as positive. When no agglutination was seen, KAtex was considered negative.

Data analysis

Numerical variables were summarized by mean and SD if normally distributed and if they were not, by median and quartiles. Mean values were compared with Student's *t*-test and medians with the Mann–Whitney *U*-test, at a critical α-level of 0.05. All *P*-values were two-sided. The results of KAtex obtained in ITMA were used to assess diagnostic accuracy of the KAtex. Sensitivity of KAtex was assessed in confirmed kala-azar patients, i.e. those who were parasitologically positive. The specificity of KAtex was assessed in the group of patients with negative parasitology and a negative DAT (i.e. DAT titre ≤1:3200). We excluded patients who could not be categorized according to this reference standard from the data analysis. Exact 95% binomial confidence intervals were computed for the sensitivity and specificity. The association of KAtex positivity in kala-azar patients with size of spleen, duration of fever and the parasite intensity was assessed by Pearson's chi-square for linear trend. Reproducibility between the KAtex performance at ITMA and BPKIHS was assessed by Cohen's kappa. The data were analysed with SPSS for Windows version 10.0.5 (SPSS Inc., Chicago, IL, USA).

Results

A total of 269 kala-azar suspect cases were enrolled between November 2000 and January 2002. Eight cases had to be excluded, as the KAtex could not be performed in Antwerp because urine samples were lost during transport. Of the remaining 261 cases, there were 155 confirmed kala-azar cases (with positive microscopy) and 77 non-kala-azar cases (negative microscopy and negative DAT). Twenty-nine cases were excluded from the analysis, 28 because they could not be classified by our reference standard (negative microscopy but positive DAT) and one because he left the hospital early, against medical advice. The diagnosis of kala-azar in the 155 patients was reached by a positive bone marrow ($n = 152$) or positive spleen aspirate ($n = 3$). The quality control done on the parasitology smears showed perfect concordance for grade 2+ or above, but for smears with scanty parasites (grade 1), there was considerable discrepancy.

Table 1 shows the characteristics of the kala-azar and non-kala-azar cases. There were significant differences between the two groups with respect to the duration of fever, spleen size, haemoglobin percentage, platelet count and the white cell count.

The most frequent discharge diagnosis in the non-kala-azar group was malaria (42 patients, 54.5%). The other diagnoses were haematological malignancy ($n = 8$), tuberculosis ($n = 6$), haemolytic anaemia ($n = 4$), portal

Table 1 Clinical and haematological characteristics of confirmed kala-azar and non-kala-azar patients in Dharan, Nepal

	Confirmed kala-azar ($n = 155$)	Non-kala-azar ($n = 77$)	*P*-value†
Age (years)‡	23 (13; 26)	20 (10; 30)	0.084
Fever duration (weeks)‡	8 (4; 12)	4 (3; 8)	0.001
Spleen size (cm)‡	5 (3; 8)	3 (1; 5)	<0.001
Haemoglobin (gm%)*	8.4 (2.0)	9.5 (2.7)	<0.001
Median WCC (mm^3)‡	3800 (3100; 4400)	6050 (4550; 10 150)	<0.001
Platelets (mm^3)*	140 143 (67 632)	237 947 (131 727)	<0.001

* Mean (SD).
† Mean values were compared with Student's *t*-test, medians with a Mann–Whitney non-parametric test.
‡ Median (quartile 1, quartile 3).

hypertension ($n = 3$), enteric fever ($n = 3$), septicaemia ($n = 3$), HIV ($n = 3$), systemic lupus erythematosus ($n = 1$) and other infections ($n = 4$).

The KAtex performed at ITMA was positive in 74 of the 155 kala-azar patients, sensitivity 47.7% (95% CI: 39.7–55.9) and negative in 76 of the 77 non-kala-azar patients, specificity 98.7% (95% CI: 93–100). The association of the intensity of parasite, size of spleen and duration of fever with KAtex sensitivity in the 155 confirmed kala-azar patients was assessed. As shown in Table 2, the sensitivity of KAtex increased significantly with increasing parasite intensity, spleen size and duration of fever.

In Nepal all urine samples were tested within 3 months of collection. In Antwerp, storage time varied, but all samples were analysed within 26 months. We found no significant difference in the sensitivity of samples stored longer or shorter than 10 months.

Table 2 KAtex sensitivity according to duration of fever, spleen size and parasite grading in 155 confirmed kala-azar patients in Dharan, Nepal, 2001–2002

	N	Number of KAtex-positive	KAtex sensitivity	*P*-value
Duration of fever (weeks)				0.024*
<9	96	39	0.406	
≥9	59	35	0.593	
Spleen size (cm)				0.005**
<4.0	44	13	0.295	
4.0–5.9	36	20	0.556	
6.0–7.9	28	11	0.393	
≥8.0	47	30	0.638	
Parasite grading				<0.001**
1	29	6	0.207	
2	43	11	0.256	
3	41	24	0.585	
4	36	28	0.778	
5	6	5	0.833	

* Pearson χ^2 test; ** χ^2 for linear trend.

In comparing the reproducibility of the tests performed at BPKIHS and ITMA, the test was negative at BPKIHS in 16 patients of the 75 that were positive at ITMA. There were also 16 negatives in ITMA from the 75 that were positive at BPKIHS. The kappa score was 0.685 (95% CI: 0.586–0.784).

Discussion

In this study, the sensitivity of KAtex was found to be low in confirmed VL cases although the specificity was excellent amongst a control group of patients with similar symptoms in whom kala-azar was ruled out. The study design purposefully included this spectrum of patients, as they would represent the persons on whom physicians would use the test for diagnosis in future.

We observed a significant increase in the KAtex sensitivity with the duration of fever, spleen size and the parasite intensity in the tissue aspirate; the first two probably reflect the duration of the illness. Parasite intensity is probably well-correlated with antigen load in urine. The low overall sensitivity is in contrast to earlier results by Attar *et al.* (2001) and unpublished data from Muzzafarpur, India and Sudan. However, Attar *et al.* (2001) reported data from Brazil where only 16 of 25 confirmed kala-azar showed a positive KAtex (64%, 95% CI: 42.5–82).

Low sensitivity in our study could possibly be explained by shorter duration of the disease in the patients presenting. Also, our series of kala-azar patients contained a high number with low parasite intensity, 46.5% had grade 1+ or 2+. On the contrary, smaller sample sizes in the earlier studies lead to wider confidence intervals around those

sensitivity estimates. Deterioration of antigen due to freezing and storage of the urine samples is unlikely to be a major contributor to this low sensitivity, as the detection of antigen was shown to be stable, although at a reduced score, over an 8 year storage period at −20 °C. Also, we observed similar low sensitivity of KAtex in a recent study comparing fresh and frozen urine samples collected from a group of kala-azar patients from Nepal (unpublished data).

The reproducibility of KAtex results was good. The main difficulty in reading the test is the discrimination of a 1+ test result (the test is normally graded from 1+ to 3+) from a negative result; any tendency to interpret the KAtex test result conservatively will decrease the sensitivity of the test.

Sensitivity is one of the crucial parameters in the choice of a diagnostic test for VL. Although not ideal as a diagnostic test in its present format, there are field settings with minimal laboratory infrastructure where the test could be of use. Given its high specificity, the positive predictive value of a positive KAtex result is likely to be high, in this study it was 0.987 (95% CI: 0.928–1.0). One might consider treating a clinical suspect patient with a positive KAtex without the need for a parasitological diagnosis. However, a negative KAtex result is of little value, and such a patient should be referred for further investigation.

A latex agglutination test detecting a heat stable leishmanial antigen from the urine from kala-azar patients presents an interesting technology. It is simple to use, results are immediately available, it does not require any electric appliances and is thus feasible in the rural health centres. Testing the urine is acceptable to the patients especially when compared with the alternative of the invasive bone marrow aspirations. Testing of an antigen has moreover a potential for monitoring response to treatment where the antibody-based tests are of no help. Therefore, the test merits further development and evaluation. However, future assessment of KAtex should carefully document the stage of disease, parasite intensity as well as handling of samples (fresh/stored).

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Field validity, reproducibility and feasibility of diagnostic tests for visceral leishmaniasis in rural Nepal

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Summary

OBJECTIVES To assess the field accuracy, reproducibility and feasibility of the formol gel test (FGT), the urine latex agglutination test (KAtex) and a rK39 antigen-based dipstick for the diagnosis of visceral leishmaniasis (VL) in rural Nepal.

METHOD Patients with clinical suspicion of VL were recruited at Rangeli District Hospital (DH), a 15-bed government hospital located in south-eastern Nepal. FGT, KAtex and rK39 dipstick tests were performed on site and later repeated at a reference kala-azar diagnostic laboratory to assess reproducibility. Diagnosis of VL was confirmed by either a positive bone marrow aspirate examination or a positive direct agglutination test (DAT titre ≥1:3200) in patients who later responded to anti-leishmanial therapy.

RESULTS Of 155 patients initially recruited, 142 (85 with VL and 57 with another diagnosis) were included in the study. The sensitivity of the rK39 dipstick [89%; 95% confidence interval (CI): 81–94] was significantly higher than that of the KAtex (57%; 95% CI: 46–67) and the FGT (52%; 95% CI: 41–62). All three tests had a specificity of at least 90%. Agreement was higher for the rK39 dipstick ($\kappa = 0.87$) than for the FGT (0.68) and the KAtex (0.43). All tests required ≤20 min of actual work and ≤40 min to obtain the results.

CONCLUSION The rK39 dipstick was easy to do, more accurate and reproducible than other rapid diagnostic tests for VL in a DH of rural Nepal. It should be integrated into the field diagnostic algorithm of VL in this region and mechanisms to secure its availability should be found.

keywords kala-azar, visceral leishmaniasis, diagnosis, serology, antigen detection, Nepal

Introduction

Visceral leishmaniasis (VL) or kala-azar affects an estimated 500 000 persons yearly, predominantly in the poor rural areas of India, Bangladesh, Nepal, Sudan and Brazil (Desjeux 1996). Most patients present with prolonged fever, weight loss and an enlarged spleen. This clinical picture is shared by several other endemic diseases such as malaria, disseminated tuberculosis or enteric fever, which are also commonly seen in the same focus. Laboratory testing is therefore necessary to confirm the diagnosis of VL. A highly sensitive and specific diagnostic approach is needed, because of the fatal evolution of the disease without specific treatment and the serious toxicity of the most widely used first-line drug, sodium stibogluconate (Sundar *et al.* 2000). Most communities affected by VL are poor and located in remote rural areas with limited access to referral hospitals. Therefore, laboratory tests for VL diagnosis need to be cheap, easy to perform and available in peripheral health centres to insure that most patients have an adequate access to diagnosis. The development of diagnostic tests for improved case-management of VL has been rated as one of the most needed among other infectious diseases prevalent in the developing world (Mabey *et al.* 2004).

Direct microscopic examination of spleen aspiration is considered the gold standard for VL diagnosis but the expertise required for the procedure makes it unsuitable for generalized field use. Alternatively, lymph node or bone marrow aspirates are often used but these are substantially less sensitive (Zijlstra *et al.* 1992). Serological tests have been developed to replace parasitological diagnosis in the

field. The direct agglutination test (DAT), developed in the 1980s (Harith *et al.* 1986, 1987), has been validated in several endemic areas (Boelaert *et al.* 1999a, b, c, 2004). Its use has been encouraged by the World Health Organization (WHO) for surveillance and control programs of VL (DAT workshop, Antwerp, 25–27 March 1998). Unfortunately, the relative sophistication of the DAT procedure (e.g. need for micropipettes and microtitration plates) restricts its use to referral hospitals or well-supported health centres.

Simpler tests designed for field use exist. Recently, serological testing based on the detection of antibodies against a recombinant antigen derived from a 39-amino acid repeats in *Leishmania chagasi* (rK39) was developed into a dipstick format. Validation studies have shown variable results depending on the location of the study site, the brand of the dipstick and the study method (Sundar *et al.* 1998; Delgado *et al.* 2001; Zijlstra *et al.* 2001; Chappuis *et al.* 2003; Schallig *et al.* 2002; Carvalho *et al.* 2003; Veeken *et al.* 2003; Boelaert *et al.* 2004). The formol gel test (FGT) is a cheap, easy to perform but poorly sensitive test that is at present the only diagnostic test available for VL in many peripheral health centres in the Indian sub-continent and in East Africa (Chowdhury *et al.* 1992). A urinary antigen-based latex agglutination test (KAtex) has been recently developed and evaluated. The specificity of KAtex was found to be excellent but its sensitivity varied from 48% to 100% (Attar *et al.* 2001; Rijal *et al.* 2004).

In Nepal, where VL is endemic in 12 south-eastern districts located in the Terai region, the DAT, rK39 dipstick, FGT and KAtex have been prospectively evaluated in groups of clinical suspect patients recruited in referral tertiary hospitals (Chappuis *et al.* 2003; Boelaert *et al.* 2004; Rijal *et al.* 2004). However, the performance of the tests might be different in a 'real life' setting of district hospitals (DHs), where most of the VL patients attend, for several reasons. First, patients consulting in a DH may present with a less advanced disease, thus modifying the test's sensitivity and specificity. For example, the KAtex urine test was shown to be significantly less sensitive in patients with a shorter duration of fever and a smaller spleen size (Rijal *et al.* 2004). Second, the prevalence (prior probability) of VL among clinical suspects can differ from the one found in a referral hospital and this would directly influence the positive and negative predictive value of the tests. Third, limited training of the laboratory technicians, their high workload and poor logistic facilities can alter the execution and/or the interpretation of the test. For these reasons, we assessed the validity, reproducibility and feasibility of three candidate tests for decentralized use, the rK39 dipstick test, the FGT and the KAtex, in the setting of a first-referral hospital located in an endemic area of VL in Nepal.

Materials and methods

Study site

The study was conducted at Rangeli DH in Rangeli, a 15 000 inhabitants town of the district of Morang. This rural hospital serves a district with 800 000 inhabitants located in the Eastern Region of Nepal and bordering the Bihar State of India. The Rangeli DH is a 15-bed government hospital that caters to patients from neighbouring villages who may, or may not, have had a prior consultation at the health post in their village. The clinical activities at Rangeli DH are supervised by two medical doctors. The other medical staff includes one clinical officer, four nurses, one laboratory technician and one laboratory assistant. The two laboratory workers underwent a 2-week training before the start of the study at the Kala-azar Laboratory of the B.P. Koirala Institute of Health Sciences (BPKIHS). Both were trained in performing and interpretation of the rK39 dipstick and the KAtex tests and reviewed the procedures of the FGT and the microscopic search for *Leishmania donovani* bodies in bone marrow aspirates. The BPKIHS is a 648-bed teaching hospital with strong expertise in VL diagnostics and research located in Dharan, at 150 km by road from Rangeli DH. The BPKIHS laboratory served as a reference laboratory in this study.

Patients

All patients with a history of 2 weeks or more of fever and a clinically assessed enlarged spleen were defined as suspect VL cases and were eligible for the study. Patient recruitment aimed at enrolling at least 50 VL cases and 50 non-VL cases to achieve sufficient precision for the sensitivity and specificity estimates of the tests. Therefore the total sample size was fixed at 150 patients, assuming a certain degree of imbalance between VL and non-VL cases. Patients were enrolled consecutively until the required sample size was achieved. They were included in the study after informed consent given by the patient or his/her guardian in case of minors. The research protocol was approved by the Ethical Committee of the BPKIHS in July 2001.

Diagnostic work up

All patients included in the study were admitted in the in-patient ward of the Rangeli DH for the initial diagnostic

work up (Figure 1) and treatment. Blood was drawn on the day of admission (day 0) for a complete blood count and a thick and thin smear for malaria parasite. Two filter papers were impregnated with whole blood, allowed to dry and transported to the Kala-azar Laboratory of BPKIHS within 48 h. After elution of the blood from the filter papers, the DAT was performed at the BPKIHS and the result was faxed back to the physician of Rangeli DH. The remaining blood sampled on day 0 was centrifuged at Rangeli DH and the serum was collected to perform the FGT and the rK39 antigen based dipstick. Urine was collected from all patients and stored in the refrigerator between 4 and 8 °C for a maximum of 48 h. A bone marrow aspirate was performed on day 0 for all patients and six slides were prepared and fixed. Slide staining and a microscopic search for the amastigote form of *L. donovani* were carried out independently at the Rangeli DH Laboratory and at the Kala-azar Laboratory of the BPKIHS. Quantification of parasites was graded from 1+ to 6+. Slides with discrepant reading results between the two laboratories were reviewed at the Kala-azar Laboratory of the BPKIHS and the outcome of this additional reading was recorded as final result. Testing for HIV was not performed because of the lack of proper counselling available at Rangeli DH and the expected low prevalence of HIV among the rural population living in the area.

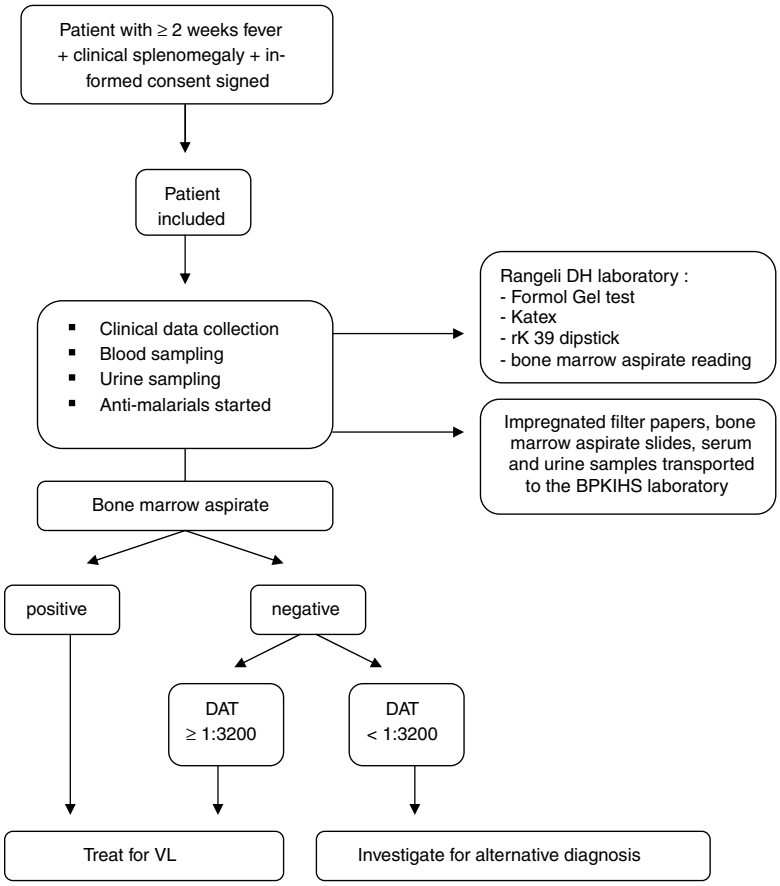

Figure 1 Operational flow-chart for the diagnosis of VL at the Rangeli DH, Nepal, during the study period (September 2001–December 2002).

Test procedures

For the FGT, 20 μl of 40% formaldehyde (Glaxo India Ltd., Bombay, India) was added to 200 μl of the patient's serum in a glass tube. After 20 min, the gelification reaction was visually assessed as positive or negative.

The rK39 antigen-based dipstick (Insure One-Step Rapid Test for VL, InBios International, Seattle, USA) was performed according to the manufacturer's instructions. In brief: 20 μl of the patient's serum was added to the test's strip. The strip was placed in a test tube and two drops of the chase buffer solution were added. The test was read after 5 min and, if negative, after 10 min. Even a weak line was considered as positive.

The Latex agglutination test (KAtex, Kalon Biological Ltd., Aldershot, UK) was performed according to the manufacturer's instructions. As pre-treatment, 1 ml of urine was transferred into the sample tube and placed in a rack immersed in a boiling water bath for 5 min. The sample and the reagents were brought to room temperature before testing. About 50 μl of the treated urine sample were added onto a reaction zone on the glass slide and a drop of test latex was added to it. The liquids were stirred to a completely homogenous mixture and rotated continuously for 2 min. Any agglutination discerned when compared with the negative control was considered as positive.

Formol gel test, rK39 dipstick and KAtex were all performed on day 0 in Rangeli DH laboratory. Results were kept blinded to the physician in charge of the patient. The remaining serum and urine were stored in the refrigerator and sent in a cool box up to three-time per week to the Kala-azar Laboratory of the BPKIHS where all tests were repeated by a laboratory technician with extensive experience in VL diagnosis within 24 h after reception. The same test procedures were used in both laboratories.

The time interval between the sampling of blood/bone marrow and the test results, as well as the time required to perform the tests, were measured in a subset of nine patients at the Rangeli DH laboratory by an independent observer. The feasibility of the diagnostic tests was also qualitatively assessed with the laboratory workers of the Rangeli DH. The cost per test unit was determined by the price in use at Rangeli DH for the FGT and the bone marrow aspiration and reading. The cost of the rK39 dipstick was recorded from the retail price of the manufacturer. The commercial cost of the KAtex has not been determined yet.

Treatment and follow up

Malaria is most frequently due to *Plasmodium vivax* in this region and blood-circulating parasites are usually scanty, leading to possible false negative blood smear examination. Therefore, all patients included in the study received a full course of anti-malarials on admission day, independently of diagnostic tests results. Early specific treatment also allowed for an easier assessment of response to anti-malarials during the short period of hospitalization. All patients with a positive bone marrow aspirate or a negative bone marrow aspirate but a positive DAT result (end-dilution titre ≥1:3200) were treated with intramuscular sodium stibogluconate (SAG from Albert David Ltd., Calcutta, India) 20 mg/kg/day for 30 days. The initial injections were performed at Rangeli DH until clinical improvement and the remaining injections were completed in the village health post. Patients with a previous history of treatment for VL were considered as relapse cases and were treated with intravenous amphotericin B 0.5 mg/kg/day for 14 days. Patients with a negative initial bone marrow aspirate and negative DAT were further investigated at Rangeli DH (or referred to the BPKIHS) and treated for an alternative diagnosis.

All VL and non-VL patients were scheduled for follow-up visits at 1, 3 and 6 months after hospital discharge. Bone marrow aspiration was repeated at 1 (test of initial cure) and 6 month (test of definite cure) follow-up visit for all patients with a positive initial bone marrow aspirate and at any visit in case of clinical suspicion of VL.

Case definitions

As the sensitivity of bone marrow aspirate is only around 80%, we based our reference standard on a combination of parasitological, serological (DAT) and clinical evidence. A confirmed case of VL was defined as a patient with (1) a positive bone marrow aspirate during initial evaluation or (2) a positive bone marrow aspirate during any follow-up visit of an initial aspirate-negative case or (3) a negative bone marrow aspirate but a positive DAT (titre ≥1:3200) at day 0 and an absence of response to anti-malarial treatment but a successful response to anti-leishmanial therapy (definite cure documented at month 6 of follow up). A confirmed case of non-VL was defined as a patient with a negative bone marrow aspirate at initial and follow-up evaluation with (1) a negative DAT (titre <1:3200) or (2) a positive DAT but a definite cure with anti-malarial or other non-VL specific therapy. All other patients were classified as cases with uncertain disease status and were excluded from the analysis.

Data analysis

Personal and medical characteristics, diagnostic tests' results, follow-up data and patients' outcome were entered

in case-report forms by the study field supervisor. Data from the case-report forms were entered in an Excel data sheet and later checked by two investigators. The data were analysed with SPSS 11.0 for Windows version (SPSS Inc., Chicago, IL, USA). Numerical variables were summarized by mean and SD if normally distributed and if they were not, by median and quartiles. Categorical variables were compared using cross-tabulations and chi-square tests whereas numerical variables (means) were compared with Student's *t*-test, at a critical α-level of 0.05. All *P* values were two-sided. Sensitivity, specificity, positive and negative predictive values and their exact 95% binomial confidence interval were calculated for the FGT, the rK39 dipstick test and the KAtex from the groups of confirmed VL and non-VL patients. Reproducibility between the performance of the FGT, rK39 dipstick, KAtex and bone marrow aspirate at the Rangeli DH and BPKIHS was assessed by Cohen's kappa and interpreted following the grading system described by Landis and Koch (1977). The time to get the test result was measured from the sampling of blood or bone marrow to the availability of the test result. The time of actual work for the nurse or physician (blood or bone marrow sampling) and the laboratory workers (test procedures) was measured, including working times of 2 min for blood centrifugation, 2 min for the FGT procedure and 10 min for staining of bone marrow aspirates.

Results

Between September 2001 and December 2002, 155 patients were admitted to Rangeli DH with a clinical suspicion of VL. Of these, 154 patients were recruited in the study and one patient defaulted. Four patients did not complete the diagnostic work up and were excluded from the analysis. Of the 150 remaining patients, the proportion of patients examined during follow-up 1, 3 and 6 months after hospital discharge was 64%, 55% and 72%, respectively. After completion of the diagnostic work-up, treatment and follow-up, 85 patients (57%) were confirmed with VL whereas 57 patients (38%) had VL excluded and were classified as non-VL patients. Of the 85 patients with VL, the diagnosis was confirmed by a positive bone marrow aspiration in 75 patients during initial evaluation and in four patients during follow-up while six patients had a DAT ≥1:3200 and were definitely cured 6 months after completion of sodium stibogluconate treatment. The final diagnosis of the 57 non-VL patients was malaria (38), enteric fever (10), tuberculosis (4), liver disease (2), haematologic disorder (1) and others (2). Fifty-two (91%) non-VL patients had a negative initial DAT and did not develop VL during follow up. Five (9%) non-VL patients had a positive DAT but did not receive anti-leishmanials and were definitely cured at 6 months after treatment for malaria (3), enteric fever (1) and tuberculosis (1). Eight patients (5%) had an uncertain diagnosis and could not be classified as VL or non-VL: all had a DAT ≥1:3200 but did not receive SAG treatment and/or were lost to follow up. These eight patients were excluded from further analysis.

The general, clinical and biological characteristics of the 85 VL patients and the 57 non-VL patients are presented and compared in Table 1. Weight loss, emaciation and darkened skin were significantly more frequent in patients with VL ($P < 0.05$). VL patients also presented with a significantly longer duration of fever, a larger spleen and lower Hb, WBC and platelet counts ($P < 0.05$).

Test validity

The three tests were performed at the Rangeli DH Laboratory in all 142 patients. Results of the FGT and the rK39 antigen based dipstick were all included in the validation analysis. One month after the start of the study, we found that the reagent initially supplied with the KAtex (Lot No. K11-035/1) had a defect (absence of reaction with the positive control at both Rangeli DH and BPKIHS). The reagent was swiftly replaced by the manufacturer. Only the results of the KAtex from the 124 patients tested with the new lot of reagent (Lot No. K11-064/1) were included in the validation analysis.

The FGT had a 52% sensitivity [95% confidence interval (CI): 41–62], a 97% specificity (95% CI: 88–99), a 96% PPV (95% CI: 86–99) and a 57% NPV (95% CI: 47–67). The rK39 antigen based dipstick test had a 89% sensitivity (95% CI: 81–94), a 90% specificity (95% CI: 79–95), a 93% PPV (95% CI: 85–97) and a 85% NPV (95% CI: 74–92). The KAtex urine test had a 57% sensitivity (95% CI: 44–67), a 98% specificity (95% CI: 88–100), a 98% PPV (95% CI: 89–100) and a 56% NPV (95% CI: 45–67). The performance of the FGT and the rK39 dipstick test, when used in combinations, are also shown in Table 2.

The sensitivity and the specificity of the tests were not significantly modified when the analysis was restricted to the 130 patients with no previous history of VL (data not shown). Interestingly, out of the six non-VL patients with a history of previous VL, all had a negative FGT and KAtex but two patients had a falsely positive rK39 dipstick. These two patients also had a positive DAT with high titres (≥1:102400).

Test reproducibility

The FGT, rK39 dipstick and KAtex urine tests were repeated using the same test batches at the Kala-azar

Table 1 Comparison of the general, clinical and biological characteristics of the 85 VL patients and the 57 non-VL patients at the Rangeli DH, Nepal

Characteristics	VL patients (total: 85) *n* (%) or mean (SD)	Non-VL patients (total: 57) *n* (%) or mean (SD)	*P* value
Gender			
Female	37 (43)	20 (35)	0.31
Male	48 (57)	37 (65)	
Age	22.0 (13.4)	25.5 (15.2)	0.15
Profession			
Farmer	24 (28)	19 (33)	0.34
Student or pre-school	33 (39)	18 (32)	
Housewife	18 (21)	16 (28)	
Others	10 (12)	4 (7)	
Previous treatment for VL	6 (7)	6 (11)	0.47
Symptoms (self-reported)			
Chills	31 (37)	26 (46)	0.28
Skin darkening	27 (32)	3 (5)	<0.001
Anorexia	21 (25)	9 (16)	0.20
Cough	13 (15)	6 (11)	0.41
Weight loss	13 (15)	2 (4)	0.025
Abdominal pain	3 (4)	0 (0)	0.15
Bleeding	0 (0)	0 (0)	–
Duration of fever (weeks)	6.3 (4.8)	3.6 (2.6)	<0.001
Signs			
Emaciation	28 (33)	4 (7)	0.001
Hepatomegaly	79 (93)	49 (86)	0.17
Enlarged lymph nodes	3 (4)	2 (4)	0.99
Spleen size from costal margin (cm)	6.4 (3)	4.5 (2.8)	<0.001
Haematology			
Haemoglobin (g/dl)	9.2 (1.3)	9.7 (1.2)	0.01
White blood cell count (per mm^3)	4270 (1193)	6880 (2180)	<0.001
Platelet count (per mm^3)	126753 (34746)	159404 (31913)	<0.001

VL, visceral leishmaniasis.

Laboratory of the BPKIHS in 135, 142 and 116 patients, respectively. The strength of agreement was almost perfect for the K39 dipstick test ($\kappa = 0.87$), substantial for the FGT ($\kappa = 0.68$) and moderate for the KAtex ($\kappa = 0.43$).

Despite the fact that microscopic reading of bone marrow aspirates was performed on different sets of slides (but sampled at the same time) at Rangeli DH and BPKIHS, we assessed the strength of agreement and we found it to be substantial ($\kappa = 0.71$). Twenty (14%) out of 142 sets of slides examined showed discordant results, of which 17 (85%) were found in slides graded as 1+ by one of the readers.

Test feasibility

The cost per test unit, time of actual work and time to get the results are presented in Table 3. The median time of actual work was below 10 min for the FGT and the rK39 dipstick, 20 min for the KAtex urine test and 50 min for the bone marrow aspirate whereas the median time to get the results was 20 min for the rK39 dipstick, 25 min for the KAtex, 40 min for the FGT and 80 min for the bone marrow aspirate.

Discussion

We found the rK39 antigen-based dipstick (Insure one-step rapid test for VL) to be significantly more sensitive and more reproducible than the FGT and the urine antigen detection test (KAtex). Moreover, the rK39 dipstick required little actual work and gave quick results. The FGT and the KAtex were insufficiently sensitive but showed a non-significant trend towards a higher specificity than the rK39 dipstick.

An accurate validation of diagnostic tests for VL had been previously achieved in several studies conducted in optimally controlled conditions at the BPKIHS, a referral teaching hospital located in Eastern Nepal (Chappuis *et al.* 2003; Boelaert *et al.* 2004; Rijal *et al.* 2004). Our study was conducted in the real conditions of a DH located in the

Table 2 Sensitivity, specificity, positive and negative predictive values of the FGT, the K39 antigen-based dipstick test and the KAtex urine test at the Rangeli DH, Nepal

Diagnostic test	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
Individual tests				
FGT	52 (41–62)	97 (88–99)	96 (86–99)	57 (47–67)
K39 dipstick test	89 (81–94)	90 (79–95)	93 (85–97)	85 (74–92)
Katex urine test	57 (46–67)	98 (88–100)	98 (89–100)	56 (45–67)
Tests in combination				
FGT+and/or K39 dipstick+	93 (85–97)	88 (77–94)	92 (84–96)	89 (79–95)
FGT+ and K39 dipstick+	47 (37–58)	98 (91–100)	98 (87–100)	55 (46–65)

FGT, formol gel test; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

Table 3 Cost per unit, time of actual work and time to obtain results of the diagnostic tests performed at the Rangeli DH, Nepal

Diagnostic test	Cost per unit (US $)	Time of actual work (min)*	Time to obtain results (min)*
rK39 dipstick test	1†	9 (6.5; 9)	20 (15; 25)
FGT	0.25‡	6 (6; 7)	40 (34; 57.5)
Katex urine test	Undetermined	20 (15; 22.5)	25 (20; 27.5)
Bone marrow aspirate	1.5‡	50 (45; 55)	80 (71.2; 97.5)

FGT, formol gel test.
* Median (quartile 1; quartile 3).
† Price communicated by the manufacturer.
‡ Price billed to the patients at Rangeli DH.

same endemic area, and we evaluated the effectiveness rather than the efficacy of those diagnostic tests. No extra-staff was hired for the study and the laboratory technicians received a limited previous training on the diagnostic test procedures. This might have impaired the accuracy of the test validation but we believe that our results showed a more realistic picture of the performance of the tests when used in daily field practice. Our reference standard for the validation was based on a combination of parasitological, serological and clinical evidence, as systematic splenic aspiration of all suspects was not possible in this context. Nonetheless this should not jeopardize the validity of our sensitivity and specificity estimates. Documented response to treatment in chronic febrile splenomegaly patients with a positive DAT should be considered as sufficient evidence of VL (Zijlstra *et al.* 1991), because antimonials have a very narrow therapeutic spectrum.

Despite these limitations, the results of the tests validation were in accordance with previous studies (Boelaert *et al.* 2004; Rijal *et al.* 2004), which equally showed a low sensitivity of FGT and KAtex. This low sensitivity is most likely influenced by patient's delay in presenting at the rural hospital. Indeed, we found no clear differences in the clinical and laboratory parameters when comparing to cohorts of VL patients previously studied at the tertiary care centre BPKIHS (data not shown), reflecting a similar degree of disease progression. However, the sensitivity of KAtex appears better in VL patients co-infected with HIV because of their higher parasite load (Riera *et al.* 2004).

The high specificity of KAtex also confirmed previous reports and highlights the very interesting concept of the test (detection of a specific antigen). Further developments to improve the sensitivity of specific antigen detection in the urine are underway. The high specificity of the FGT was more surprising considering the low intrinsic specificity of the test principle (detection of an increased level of polyclonal immunoglobulins), but confirmed an earlier report from Nepal (Boelaert *et al.* 2004). This should be confirmed in other VL endemic areas such as East Africa where polyclonal hypergammaglobulinemia might be more commonly found with other diseases like *Plasmodium falciparum* infections (Abele *et al.* 1965).

The rK39 dipstick (Insure one-step rapid test for VL) performed well in our study with sensitivity and specificity of respectively 89% and 90%. The same dipstick was previously validated in Nepal and India, showing sensitivity and specificity ranges of 90–100% and 93–100%, respectively (Bern *et al.* 2000; Sundar *et al.* 2002a,b; Boelaert *et al.* 2004). A lower specificity (71%) was found with an earlier generation of the Insure dipstick in Nepal (Chappuis *et al.* 2003). Other brands of rK39 antigen-based dipsticks have been evaluated elsewhere but are either no longer produced (Sundar *et al.* 1998; Zijlstra *et al.* 2001; Veeken *et al.* 2003), or in need of further evaluation (Brandonisio *et al.* 2002; Iqbal *et al.* 2002). RK39 antigen-based dipsticks have so far performed poorly in Sudan (Zijlstra *et al.* 2001; Veeken *et al.* 2003). A newly manufactured rK39 dipstick (Opti-LEISH from Diamed AG, Switzerland) showed promising results in India (Sundar *et al.* 2003), Uganda (personal observation) and, interestingly, in Sudan (Ritmeijer K, Melaku J, Möller M, Kipngetich S, O'Keefe C & Davidson RN, in press).

We found that two out of six non-VL patients with a history of previous treatment for VL had a false positive dipstick result. The rK39 antigen-based dipsticks are not suited for the diagnosis of relapses due to the long persistence of antibodies after initial treatment (Zijlstra *et al.* 2001), as observed with other serological tests for VL such as the DAT (Hailu 1990). For patients with a previous history of VL, parasitological diagnosis remains the approach of choice but antigen detection in the urine could be an interesting alternative, provided that the sensitivity of the test improves.

We found an almost perfect agreement of the rK39 dipstick between the Rangeli DH and the BPKIHS laboratories. Inter-reader concordance was already found to be 100% in a previous study (Schallig *et al.* 2002). This excellent reproducibility most likely reflects the simplicity of the procedure and the easy interpretation of most dipstick results. In contrast, we found only a moderate reproducibility of the KAtex urine test between the two laboratories ($\kappa = 0.43$), lower than previously reported ($\kappa = 0.69$) by Rijal *et al.* (2004). This is likely to be due to the difficulty in interpreting weakly positive agglutinations and to some practical problems occurring during the test procedure such as having the urine sample spoiled by the boiling water during the heating process. Nevertheless, we cannot exclude that the strength of concordance was underestimated by some degree of deterioration of the antigen during storage and transport of urine samples. Discordant results of bone marrow aspirate examinations between the two laboratories were not rare and mostly occurred in patients with low parasite densities, emphasizing the high level of expertise required to perform this test with accuracy.

The time to get the results of the FGT, rK39 dipstick and the KAtex urine test is equal or less than 40 min. The three tests thus deserve to be labelled as 'rapid tests'. They compare favourably with the minimal time of 18 h to get the result of the DAT when performed on site. Their direct cost is low (0.25 US $ for the FGT) to moderate (1 US $ for the dipstick). The time of actual work to perform the KAtex urine test exceeds the time needed to perform the rK39 dipstick and the FGT. This should be taken into account for the calculation of the real cost of these diagnostic tests, bearing in mind that the cost of test-treatment strategies depends mainly on the cost of hospitalization and treatment (Boelaert *et al.* 1999d).

Our findings support the recent yearly distribution of several thousands Insure dipsticks to the DHs of VL endemic areas by the Nepalese Ministry of Health. As recently shown in the neighboring Bihar State of India (Sundar *et al.* 2002b), the rK39 antigen-based dipstick appears as the best first-line single diagnostic test for the diagnosis of VL in this part of the world. It fulfils most of the 'ASSURED' criteria that describe the ideal characteristics of a diagnostic test for resource-limited settings (Mabey *et al.* 2004). The DAT is a well-validated and robust test, especially when freeze-dried antigen is used (Abdallah *et al.* 2004), but it is too sophisticated to be widely used at first line health services in the endemic areas of Nepal and India. Sending blood-impregnated filter papers to a reference laboratory performing the DAT, as done during our study and routinely in some endemic areas (i.e. Somalia), is neither practical nor sustainable. Integrating the FGT in the diagnostic tree as a first-step procedure and restraining the use of the rK39 dipsticks to negative FGT results appears as a promising approach in this endemic region. This approach would slightly improve diagnostic accuracy (cf. Table 2) and would decrease the cost by decreasing the consumption of dipsticks (a 32% decrease in our study). A careful cost-effectiveness analysis remains to be performed before a final diagnostic tree can be proposed to national policy makers. Similar evaluations should be done in other VL endemic areas such as East Africa or South America.

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Validité, reproductibilité et faisabilité sur le terrain des tests de diagnostic de la leishmaniose viscérale en milieu rural au Népal

OBJECTIFS Evaluer sur le terrain la précision, la reproductibilité et la faisabilité du test au Gel de Formol (FGT), du test de l'agglutination urine-latex (KAtex) et du test sur bandelette à l'antigène rK39, pour le diagnostic de la leishmaniose viscérale (LV) en milieu rural du Népal.

MÉTHODE Les patients avec une suspicion clinique de la LV ont été recrutés à l'hôpital du district de Rangeli, un hôpital gouvernemental de 15 lits, situé dans le sud-est du Népal. Les tests FGT, KAtex et au rK39 ont été appliqués sur place et répétés ultérieurement dans le laboratoire de diagnostic de référence du Kala-azar pour évaluer leur reproductibilité. Le diagnostic de la LV a été confirmé soit par examen positif d'aspiration de moelle osseuse ou par un résultat positif du test d'agglutination direct (titre DAT ≥1:3200), chez les patients qui, plus tard ont répondu au traitement anti-leishmaniose.

RÉSULTATS Sur 155 patients initialement recrutés, 142 (85 avec la LV et 57 avec un autre diagnostic) ont été inclus dans l'étude. La sensibilité pour le test au rK39 (89%, IC 95%: 81–94) était significativement plus élevée que celle du KAtex (57%, IC95%: 46–67) et celle du FGT (52%, IC95%: 41–62). Tous les trois tests avaient une spécificité d'au moins 90%. La concordance était plus élevée pour le test au rK39 ($\kappa = 0.87$) que pour le FGT ($\kappa = 0.68$) et le KAtex ($\kappa = 0.43$). Tous les tests nécessitaient ≤20 min de travail effectif et ≤40 min au total pour l'obtention des résultats.

CONCLUSION Les bandelettes au rK39 étaient d'utilisation facile avec des résultats plus précis et plus reproductibles que les autres tests de diagnostic de la LV dans un hôpital de district dans le Népal rural. Ce test devrait être intégré dans cette région dans l'algorithme du diagnostic sur le terrain de la LV et les mécanismes pour assurer sa disponibilité devraient être trouvés.

Mots clés kala-azar, leishmaniose viscérale, diagnostic, sérologie, détection d'antigènes, Népal

Validez en el campo, reproducibilidad y factibilidad de test diagnósticos para leishmaniasis visceral en Nepal rural

OBJETIVOS Evaluar la exactitud en el campo, la reproducibilidad y la factibilidad del test de gel de formol (FGT), el test de latex en orina (KAtex) y la tira diagnóstica basada en el antígeno rK39 para el diagnóstico de leishmaniasis visceral (LV) en el Nepal rural.

MÉTODO Pacientes con sospecha clínica de LV fueron reclutados en el hospital distrital de Rangeli, un hospital gubernamental de 15 camas, localizado en el sur occidente de Nepal. Pruebas con FGT, KAtex y las tiras diagnósticas rK39 se realizaron *in situ* y posteriormente repetidas en un laboratorio de referencia para el diagnóstico de kala-azar con el fin de evaluar su reproducibilidad. El diagnóstico de LV se confirmó, bien por aspirado de médula ósea positivo o por test de aglutinación directa (titulo DAT ≥1:3200) en pacientes que luego respondieron a terapia anti-leishmania.

RESULTADOS De los 155 pacientes inicialmente reclutados, se incluyeron 142 (85 con LV y 57 con otros diagnósticos). La sensibilidad de la tira diagnóstica rK39 (89%; 95% CI: 81–94) fue significativamente más alta que la del KAtex (57%; 95%CI: 46–67) y la del FGT (52%; 95%CI: 41–62). El acuerdo fue más alto para la tira diagnóstica rK39 ($\kappa = 0.87$) que para el FGT (0.68) o el KAtex (0.43). Todos los test necesitaron ≤20 min de trabajo real y ≤40 min para obtener resultados.

CONCLUSIÓN La tira diagnóstica rK39 fue más fácil, más exacta y reproducible que los otros test rápidos utilizados en un hospital distrital del área rural de Nepal para el diagnóstico de la LV. Se debería integrar dentro del algoritmo diagnóstico de esta región, así como encontrar los mecanismos para asegurar su disponibilidad.

Palabras clave kala-azar, leishmaniasis visceral, diagnóstico, serología, detección antigénica, Nepal

CHAPTER 5:

EFFICACY AND SAFETY OF CURRENT DRUG TREATMENT

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Treatment of visceral leishmaniasis in south-eastern Nepal: decreasing efficacy of sodium stibogluconate and need for a policy to limit further decline

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Abstract

Sodium stibogluconate (SSG) is the first-line therapy for visceral leishmaniasis (VL) in south-eastern Nepal. Recent studies from the neighbouring state of Bihar, India, have shown a dramatic fall in cure rates with treatment failure occurring in up to 65% of VL patients treated with SSG. A prospective study was conducted at a tertiary-level hospital located in south-eastern Nepal from July 1999 to January 2001. Parasitologically proven kala-azar patients with no previous history of treatment for VL were treated with SSG 20 mg/kg/d for 30 d which was extended to 40 d in those with persistent positive parasitology. Of the 110 patients who completed SSG therapy and were assessed at 1 and 6 months, definite cure was achieved in 99 patients (90%) and SSG failure occurred in 11 patients (10%). Except for the presence of hepatomegaly and a lower platelet count there was no clinical or laboratory baseline characteristic associated with treatment failure. A significantly lower cure rate (76%, $P = 0.03$) was observed in patients from the district of Saptari, which borders the antimony-resistant VL areas of Bihar. The efficacy of SSG as a first-line treatment for VL in south-eastern Nepal was still satisfactory, except for the patients living closer to the antimony-resistant VL areas of India. These findings indicate that the spread of resistance to antimonials is already taking place in Nepal and that a policy to control further spread should be urgently implemented.

Keywords: visceral leishmaniasis, *Leishmania donovani*, chemotherapy, sodium stibogluconate, drug resistance, Nepal

Introduction

Visceral leishmaniasis (VL) or kala-azar affects an estimated 500 000 persons every year worldwide, with 50% of cases reported from Bangladesh, India, and Nepal (Desjeux, 2001). Visceral leishmaniasis is considered a major health problem in Nepal and is endemic in 12 districts of the eastern and central part of the southern Teraï region, where an estimated 6 million people are at risk of acquiring the disease (Ministry of Health, Nepal, 1999/2000). This region borders the northern part of the state of Bihar in India, which has been the epicentre of the Indian epidemic.

In Nepal, as in most VL-endemic regions, the first-line treatment relies on pentavalent antimony, such as sodium stibogluconate (SSG), as recommended by the WHO (1990). In spite of the inconvenience of prolonged parenteral therapy and potential for severe adverse effects SSG is still used mainly due to its proven efficacy and, when generic compounds are used, affordability (Veeken *et al.*, 2000). However, in the state of Bihar in India, there has been an increasing resistance to SSG since the early 1980s despite a gradual increase of the dose and duration of treatment up to the maximum recommended dose of 20 mg/kg/d for 30 d (Sundar, 2001). In 1991–92, cure rates of 60% and 64% were found in 2 separate studies using this maximum recommended dose (Jha *et al.*, 1992; Sundar *et al.*, 1995). This trend has shown a further decline and a recent study has reported a long-term cure rate of only 35% (Sundar *et al.*, 2000a). Thus, SSG has now been abandoned and replaced by the more expensive amphotericin B as the first-line therapy for VL in Bihar.

In Nepal, data on the efficacy of SSG is poorly documented. There has been only 1 published study (Karki *et al.*, 1998) that compared SSG 20 mg/kg/d given for 20 d compared with 30 d in 2 groups of 27 patients each. The definite cure rate was found to be 78% and 93%, respectively.

One of the important contributing factors to the drug resistance in Bihar has been attributed to the use of infra-therapeutic doses and/or insufficient duration of SSG therapy (Sundar, 2001). This phenomenon also exists in Nepal. Moreover, the socio-cultural similarity and the open border between southern Nepal and northern Bihar facilitate cross-border population movements which may also play an important role in the spread of SSG-resistant strains of *Leishmania donovani*. The objective of the study was to obtain the current status of SSG efficacy in the VL-endemic regions of eastern Nepal and to look for any factors associated with drug resistance. A policy for the control of SSG resistance in Nepal is discussed.

Materials and Methods

Study site

This study was conducted at the B. P. Koirala Institute of Health Sciences (BPKIHS), a 650-bed University Hospital located 2 km from the town of Dharan, Sunsari district in the Eastern region of Nepal. The BPKIHS serves as a reference tertiary-level hospital for the Eastern region which includes several VL-endemic districts. Recruitment of patients took place at the outpatient department and the emergency room of BPKIHS from July 1999 to August 2000. Patients came directly to BPKIHS or were referred from the district hospitals. The Ethical Committee of BPKIHS approved the research protocol in May 1999.

Inclusion and exclusion criteria

Parasitologically proven VL cases with no history of previous treatment with SSG were included after obtaining informed consent from the patient or his/her guardian. Only patients from the 3 neighbouring districts of Sunsari, Morang, and Saptari were included as follow-up would not be practically possible for patients coming from more remote districts. There was no other exclusion criteria.

Initial evaluation

All patients with suspected VL (history of fever $\geqslant 2$ weeks duration and clinical splenomegaly) were admitted to the medical or paediatric ward for a complete examination. This included clinical evaluation, complete blood count, chemistry, blood culture, urine analysis, and chest radiography. HIV testing was done after pre-test counselling using Vironostica® and Re-

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combigen® enzyme-linked immunosorbent assay tests. Diagnosis of VL was made by Giemsa-stained bone marrow aspirate or spleen aspirate (if bone marrow aspirate was negative). Stained smears were designated as 'positive' if *L. donovani* bodies were seen or 'negative' if none were seen in 100 oil immersion fields. Two doctors read the slides independently. Parasite density score was determined microscopically in the Giemsa-stained smears by the use of a logarithmic scale ranging from 0 (no parasites per 100 oil immersion fields) to + 6 (> 100 parasites per field). For quality control, 10% of the slides were cross-checked at the Protozoology Unit (Prof. Dominique Le Ray) of the Institute of Tropical Medicine in Antwerp, Belgium.

Treatment

All the patients included were allocated into 2 treatment groups: Group A received the full treatment course in the hospital and Group B received the first 5–7 d treatment in the hospital and the rest as outpatients at the nearest health facility to their place of residence. Allocation into the 2 groups was made according to patients' preference as random allocation was not possible. Patients were treated with generic SSG (sodium antimony gluconate [SAG] from Albert David Ltd, Calcutta, India) 20 mg/kg/d for 30 d as recommended by the Nepalese National Guidelines.

Follow-up

Clinical assessment and parasitological examination for *L. donovani* bodies was repeated at the end of 30 d of SSG therapy (1-month follow-up). If *L. donovani* bodies were still present at this time, SSG was extended for another 10 d. If at the end of this extended period *L. donovani* bodies were still found in tissue aspirate, the patient was then treated with the second-line therapy amphotericin B, 0.5 mg/kg/d for 14 d. All patients were followed-up at 3 and 6 months for further clinical evaluation. Parasitological examination was repeated at 6 months in all patients and at 3 months if relapse was clinically suspected (fever, spleen enlargement). The patients were followed-up at the BPKIHS or actively in their homes if they did not attend the BPKIHS on the fixed date. All relapse cases were treated with amphotericin B 0.5 mg/kg/d for 14 d.

Case definitions

We used the following definitions: VL case, patient with clinical signs (prolonged fever and splenomegaly) and positive for *L. donovani* bodies in tissue aspirate (bone marrow or spleen); initial cure, a VL case with absence of fever and negative parasitology at the end of SSG therapy; non-responder, a VL case with positive parasitology after 40 d of SSG therapy; definitive cure, a VL case with no clinical signs of relapse and negative parasitology at 6 months follow up; relapse case, a VL case with initial cure but with reappearance of clinical signs and positive parasitology during the 6 months follow-up; and SSG failure, non-responder or relapse case.

Statistical analysis

Frequencies and cross-tabulations were used to describe the proportions of treatment failure across baseline socio-demographic and medical characteristics. Mean and median values were used for continuous characteristics such as age or duration of fever. To study the effect of place of stay, logistic regression was used to adjust for other significant socio-demographic and medical factors. Pearson's χ^2, independent sample t tests, and Mann–Whitney U tests were used to test the statistical differences across groups, where appropriate. All tests were two-tailed, with a significance level of 0.05.

Results

Between July 1999 and August 2000, 120 VL patients were included in the study and started on SSG therapy. Four patients (3.3%) died during the course of treatment: 2 from cardiotoxicity (electrocardiographic-proven arrhythmia), 1 from septic shock and 1 patient with underlying psychiatric illness who committed suicide during his hospital stay. Two other patients developed evidence of cardiotoxicity during SSG treatment and were switched to amphotericin B. The total incidence of cardiotoxicity due to SSG was thus 3.3%.

Of the 114 patients who completed 30 d SSG therapy, 103 had a negative parasitology. Eleven remained parasitologically positive and required extension of the SSG therapy to 40 d. Of these 11, only 1 became parasitologically negative and 10 remained positive after 40 d. Thus, at completion of treatment there were 104 initial cures and 10 non-responders. Four patients who had shown initial cure were lost to follow-up at 6 months. Of the 100 patients analysed at 6 months follow-up, 1 had a positive parasitology (relapse) and 99 remained parasitologically negative (definite cure).

The 10 patients with unknown status of resistance to SSG (death during treatment (4), treatment changed to amphotericin B (2), and lost to follow-up (4)) were excluded from further analysis. Of the remaining 110 patients, SSG failure (non-responders or relapse) was seen in 10% (11/110) and definite cure was achieved in 90% (99/110). Table 1 compares baseline socio-demographic characteristics, clinical signs and laboratory values in the 110 patients followed-up at 6 months.

All the patients were negative to HIV testing. A total of 84 (76%) received the full treatment as in-patients (group A) and 26 (24%) received the initial SSG treatment (5–7 d) as in-patients and the rest as out-patients in the peripheral health facilities (group B).

Treatment failure was only significantly associated with the place of stay, the presence of hepatomegaly, and a lower platelet count (Table 2). Treatment failure occurred in 24% of patients coming from the district of Saptari compared with only 5% for the district of Sunsari and 6% for the district of Morang ($P = 0.03$). Patients coming from Saptari were more frequently men (80% vs. 53%, $P = 0.02$), more frequently had a positive blood culture at the time of diagnosis (20% vs. 5%, $P = 0.01$), and had more parasites in bone marrow examination (57% with +++ to +++++ vs. 31% with + to ++, $P = 0.03$). However, patients coming from Saptari district did not show any other significant difference in disease severity such as duration of fever, spleen size, and haemoglobin count when compared with patients from the other 2 districts (data not shown). In logistic regression analysis, taking into account the effect of gender, positive blood culture and degree of parasite infestation in the bone marrow, treatment failure was still more frequent for patients coming from Saptari (OR = 6.8, 95% CI 1.3–37.1).

Discussion

In this study, we found that the current efficacy of SSG in the treatment of VL remains satisfactory in the eastern part of the VL-endemic area of Nepal where 90% of patients who completed their treatment and 6-month follow-up showed a definite cure. Except for the presence of hepatomegaly and a lower platelet count, we did not find any clinical or laboratory findings associated with resistance to SSG, as also reported by Sundar *et al.* (2000a) in India. Co-infection with HIV, an important cause of treatment failure, was not found in this study.

However, a significantly lower cure rate of 76% was found in patients coming from the district of Saptari, situated in the western extremity of the study area and closer to the northern districts of the Indian state of Bihar (Figure) where resistance to SSG is at its highest

Table 1. Comparison of baseline socio-demographic, clinical, and laboratory values in 110 visceral leishmaniasis patients treated with sodium stibogluconate in south-eastern Nepal with definite cure or treatment failure at six months follow-up, July 1999–August 2000

	Overall ($n = 110$) Mean (SD)	Definite cure ($n = 99$) Mean	Treatment failure ($n = 11$) Mean	P^a
Socio-demographic characteristics				
Age (years)	23.8 (13.9)	24.1	21.5	0.55
Clinical signs				
Duration of fever (weeks)	7.6 (7.0)	7.6	7.1	0.82
Temperature (°F)	102.3 (1.4)	102.3	102.5	0.70
Spleen size (cm)	5.7 (3.5)	5.6	6.5	0.42
Laboratory test results				
Haemoglobin (g/dL)	8.9 (1.8)	8.9	8.4	0.39
White blood cells (/mm^3)	4030 (1310)	3990	4360	0.39
Platelets (/mm^3)	148 900 (73 700)	152 400	117 300	0.02
Creatinine (mg/dL)	0.67 (0.24)	0.68	0.61	0.37
DAT end titre (1 missing value)[b]	819 200 (–)	819 200	3 276 800	0.37

[a]Independent samples *t* test.
[b]Median values and Mann–Whitney *U* test (DAT end titres were not normally distributed).

Table 2. Relationships between treatment failure assessed at 6 months follow-up and baseline socio-demographic and clinical characteristics of 110 visceral leishmaniasis patients treated with sodium stibogluconate in south-eastern Nepal, July 1999–August 2000

	Overall ($n = 110$) n (%)	Treatment failure ($n = 11$) n (%)	P^a
Socio-demographic characteristics			
Gender			0.33
Male	65 (59.1)	5 (7.7)	
Female	45 (40.9)	6 (13.3)	
Place of stay			0.03
Sunsari	38 (34.5)	2 (5.3)	
Saptari	25 (22.7)	6 (24.0)	
Morang	47 (42.7)	3 (6.4)	
Occupation			0.24
Farmer	26 (23.6)	2 (7.7)	
Housewife	22 (20.0)	1 (4.5)	
Student	50 (45.5)	8 (16.0)	
Other	12 (10.9)	0	
Symptoms and clinical signs			
Weakness	34 (30.9)	5 (14.7)	0.27
Weight loss	76 (69.1)	7 (9.2)	0.68
Cough	11 (10.0)	2 (18.2)	0.34
Abdominal pain	23 (20.9)	3 (13.0)	0.58
Vomiting	5 (4.5)	1 (20.0)	0.45
Skin darkening	31 (28.2)	4 (12.9)	0.53
Lymph nodes	5 (4.5)	0	0.45
Hepatomegaly	76 (69.1)	11 (14.5)	0.02
Laboratory test results			
BM intensity (15 missing values)			0.20
+ to ++	60 (63.2)	5 (8.3)	
+++ to +++++	35 (36.8)	6 (17.1)	
Positive blood culture	9 (8.2)	1 (11.1)	0.91
Positive malaria smear	2 (1.8)	0	0.63
Associated tuberculosis	3 (2.7)	1 (33.3)	0.17
Type of care			0.65
In-patient (Group A)	84 (76.4)	9 (10.7)	
Outpatient (Group B)	26 (23.6)	2 (7.7)	

[a]Pearson's χ^2 test.

(Sundar *et al.*, 2001). This decreased cure rate observed in patients from Saptari is not likely to be due to differences in patients' characteristics or disease severity. Our results suggest that it is most likely that the decreased response to treatment observed in Saptari district is due to resistant strains of *L. donovani* as recently demonstrated in Bihar (Lira *et al.*, 1999). The resurgence of VL in Nepal started in the early 1980s, a decade after the epidemic began in Bihar, which could explain the current observed difference of SSG efficacy between the 2 countries. The increased failure rate in patients from Nepalese districts neighbouring Bihar strongly suggests that the spread of SSG resistance is taking place in Nepal and it may follow a similar course to that in Bihar if no immediate measures are taken.

The intense cross-border population movement be-

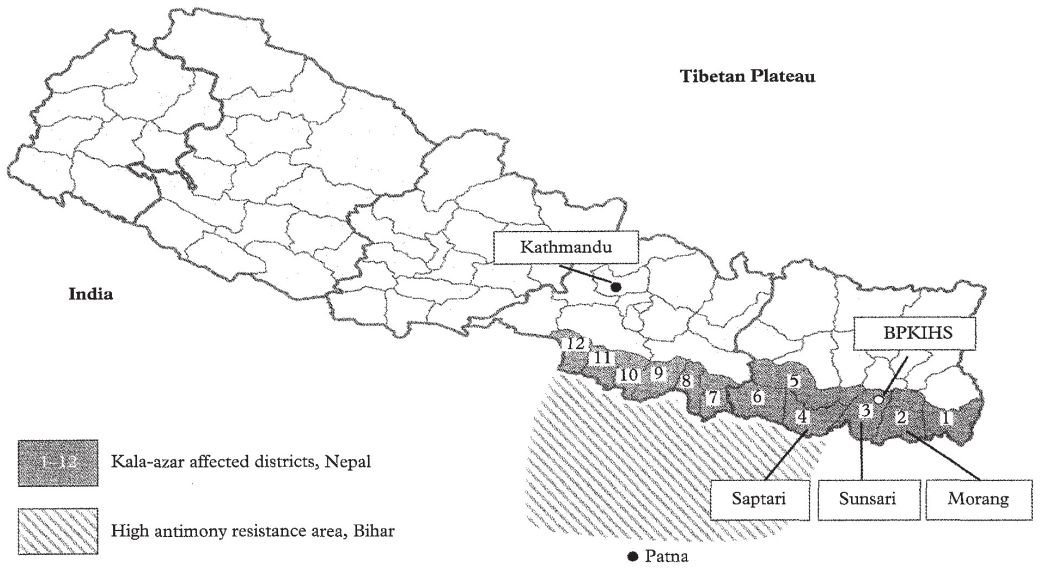

Figure. Map showing the 12 visceral leishmaniasis-endemic districts in south-eastern Nepal and the antimony resistance area in the state of Bihar, India, 1999. BPKIHS, B. P. Koirala Institute of Health Sciences.

tween the state of Bihar and the Teraï plain of Nepal could facilitate the spread of the resistant parasites. Moreover, drug misuse, a well-recognized risk factor for the development of resistant parasites (Bryceson *et al.*, 1985), is also common but poorly documented in Nepal: inadequate dosage and/or duration of treatment of SSG is often prescribed in Nepal, mostly by poorly qualified practitioners. In Bihar, Sundar *et al.* (1994) showed that only 26% of the 312 patients previously treated with SSG and presenting with a refractory disease had been treated according to the WHO (1996) guidelines.

The current free provision of VL drugs in Nepal to all hospitals located in the endemic region, as part of the National Kala-azar Control Program of the Nepalese Ministry of Health, may be a factor promoting adequate drug use. This may relieve economic pressure on patients to prematurely interrupt treatment but does not solve the constraints of transport and costs of hospitalization. Most VL patients belong to the lowest socio-economic class and have very limited capacity to pay for the cost of treating the disease (Desjeux, 1996). Moreover, the vast majority of VL patients admitted to public hospitals in Nepal receive only the initial few days of SSG until clinical improvement as in-patients and their treatment is completed as outpatients in peripheral health facilities. Considering the long daily walking time to reach health facilities, the painful i.m. injections and the other priorities of daily life, this strategy may lead to inadequate adherence to treatment, subsequently decreasing treatment efficiency and promoting the selection of resistant parasites. In our study, we did not find any significant difference in the outcome of patients between those receiving the full treatment as in-patients and those treated mostly as outpatients. However this study design had limitations, as we were unable to randomize the patients groups.

As suggested by Bryceson (2001), a policy to prevent and control the spread of SSG resistance should be implemented in VL-endemic areas like Nepal. Such a policy should include the reinforcement of current activities aiming to decrease overall disease transmission, measures to prevent drug misuse and implementation, after evaluation, of combination therapies.

The tools available for the control of anthroponotic VL have been recently reviewed by Boelaert *et al.* (2000). Vector control through insecticide spraying, in association with early case detection and treatment, is an effective way to reduce transmission of the disease (Saxena *et al.*, 1996), and thus also transmission of drug-resistant strains. This requires strong political commitment and is costly. The use of impregnated bednets has the potential to protect healthy people against VL (Bern *et al.*, 2000), and to impede untreated patients to disseminate the parasite through sandfly bites.

Measures to prevent SSG misuse in Nepal could include the widespread implementation of test-treatment algorithms, directly observed therapy for outpatients' care, education of practitioners from both public and private sectors to prescribe adequate treatment schedules, adequate supply of reliable generic SSG with proven efficacy and safety profile to health facilities in the endemic areas, and the patients' access to free or very low-cost drugs.

The use of combination therapy for VL could be an appealing approach for treating patients in drug-resistant areas, protecting each component of the combination against selection of resistant mutants and using drugs at lower and thus safer total doses. In the case of malaria, a control of mefloquine resistance has been obtained within a few years of systematic use of the artesunate–mefloquine combination in western Thailand (Nosten *et al.*, 2000). It is currently unclear if a similar strategy for VL would be successful but such an approach should be considered in areas, such as Nepal, where resistance to SSG is increasing. Combinations of SSG and paromomycin have shown to be efficient and safe in India (Thakur *et al.*, 2000), Sudan (Seaman *et al.*, 1993), and Kenya (Chunge *et al.*, 1990) but paramomycin has yet to be registered for VL treatment and its future production and cost remain unclear. Other possible combinations could include in future trials conventional or liposomal amphotericin B as well as miltefosine, an oral drug showed to be very efficient in India when used alone for 4 weeks (Jha *et al.*, 1999; Sundar *et al.*, 2000b). Miltefosine was registered for the treatment of VL in India in 2002 and a

phase IV trial will take place in India and Nepal in the coming year. However, because of the long half-life of miltefosine and the long duration of treatment, one might fear that *L. donovani* will rapidly develop resistance to this drug if it is used alone.

Regular monitoring of SSG susceptibility in Nepal should be an important component of this policy. It should be performed preferably *in vitro* using the amastigote-macrophage model, which evaluate the resistance of *L. donovani* isolates to antimonials (Croft, 2001).

The HIV seroprevalence in the general population in Nepal is currently around 0.3% but risk factors for an increasing prevalence are present (Furber *et al.*, 2002). Monitoring and fighting the progression of HIV disease is very important in this region considering the much higher risk of treatment failure in HIV-VL co-infected patients treated with SSG or with any other anti-VL drugs (Lopez-Velez *et al.*, 1998).

In conclusion, we showed that the efficacy of SSG to treat VL in eastern Nepal remains satisfactory overall but that areas of lower response to SSG exist in this region. Considering the lower price of SSG (in its generic form) and the higher cost and/or lack of availability of present alternative therapies, it is necessary that current efforts in Nepal focus on limiting the spread of resistance to SSG.

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Sodium stibogluconate cardiotoxicity and safety of generics

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Abstract

Between April 9 and May 5 2000, an outbreak of fatal cardiotoxicity occurred in Nepal amongst visceral leishmaniasis patients treated with a recently introduced batch of generic sodium stibogluconate (SSG) from GL Pharmaceuticals, Calcutta, India. Eight (36%) of 23 patients treated with this batch died, and in 5 (23%) death was attributed to the cardiotoxicity of the drug. This contrasts with the low total death rate (3.2%) and death rate due to cardiotoxicity (0.8%) observed among 252 patients treated between August 1999 and December 2001 with generic SSG from Albert David Ltd, Calcutta, India. These data show that every batch of generic SSG should be subject to rigorous quality control prior to use.

Keywords: visceral leishmaniasis, chemotherapy, sodium stibogluconate, generic drugs, cardiotoxicity, Nepal

Introduction

Visceral leishmaniasis (VL: kala-azar) remains highly endemic in many poor rural regions of India and Nepal. First-line treatment for visceral leishmaniasis in Nepal is sodium stibogluconate (SSG) 20 mg/kg/d i.m. for 30 d, as recommended by WHO. In Nepal, the Epidemiology and Disease Control Division (EDCD), Ministry of Health, distributes SSG to all hospitals managing VL patients.

As a high level of resistance to antimonials is currently being observed in the neighbouring Indian state of Bihar (Sundar *et al.*, 2000), we monitored the efficacy of SSG therapy at the B. P. Koirala Institute of Health Sciences (BPKIHS), a tertiary care centre located in Dharan, Nepal, from August 1999. For several years, the source of SSG was an Indian generic supplier (Albert David Ltd, Calcutta, India), and this drug, also called sodium antimony gluconate (SAG), has been used at BPKIHS without significant problems of toxicity or lack of efficacy. From 2 April 2000 onwards, the EDCD central pharmacy supplied generic SSG from another Indian manufacturer, GL Pharmaceuticals, Calcutta, India. At the beginning of May 2000, physicians at BPKIHS were alerted by an increase in the case–fatality rate in VL patients. The recently introduced brand of SSG was highly suspected as the potential cause of the increased mortality and it was discontinued on 7 May 2000. From that date onwards, VL patients were again treated with generic SSG manufactured by Albert David Ltd. We report the investigation of this increased mortality.

Methods

We carefully reviewed the files of all the 23 patients treated with SSG from GL Pharmaceuticals. Moreover, we also analysed the outcome for VL admitted to BPKIHS since August 1999, and reviewed the causes of death reported.

Results

Between August 1999 and December 2001, BPKIHS admitted 275 patients with parasitologically confirmed VL for treatment with SSG. All patients were admitted for at least 1 week and 42% were admitted for the full duration of treatment. Ninety-five percent of patients had a check-up performed at 1 month after completion of the drug course. Of the 23 VL patients who received 5 or more doses of SSG from GL Pharmaceuticals, 8 (36.4%) died, including 5 (22.7%) with either ECG-proven (ventricular tachycardia or fibrillation) or high clinical suspicion (cardiac arrest) of cardiotoxicity. These 5 patients received a median of 27 doses of SSG (range 8–34). Of the other 3 patients (13.6%) who died, the cause of death was bacterial meningitis in 1 and sepsis with bleeding in the other 2. In contrast, during a 2.5-year period, of the 252 patients treated with SSG from Albert David Ltd, only 8 (3.2%) had a fatal outcome (Figure). Of the 8 deaths, 2 (0.8%) were attributed to cardiotoxicity. The relative risk of death in patients treated with SSG from GL Pharmaceuticals compared with patients treated with SSG from Albert David Ltd was 11.0 (95% CI 4.5–26.5).

Discussion

The risk of fatal complications in patients treated with SSG for leishmaniasis should not be underestimated. Whereas SSG is considered safe at the WHO recommended dosages by Berman (1988), this mainly refers to patients treated for cutaneous or mucocutaneous leishmaniasis. In Indian VL patients, death due to cardiotoxicity was recently reported in 5.9% of patients (Sundar *et al.*, 2000). Visceral leishmaniasis patients frequently present with an altered general condition including malnutrition and superimposed bacterial infections. Cardiotoxicity is more likely in these patients, possibly because of concomitant electrolyte disturbances, micronutrient or vitamin deficiencies. An outbreak of cardiotoxicity has already been reported in India where 3 of 8 patients treated with generic SSG died due to an excessively high osmolarity (Sundar *et al.*, 1998). Unfortunately, analysis of the content of the suspected batch of SSG from GL Pharmaceuticals used at BPKIHS could not be performed because all remaining vials were rapidly called back and destroyed by the EDCD in Kathmandu to prevent its further use in the endemic region. The increased mortality observed at BPKIHS in April and May 2000 was also observed concomitantly in several other hospitals in Nepal. Interestingly, a similar problem involving the same SSG manufacturer seemed to have occurred also in India during the same period as the 10 June 2000 edition of an Indian daily newspaper '*The Statesman*' published a front-page article entitled 'The return of the killer injection' (Chakraborty, 2000).

The production of SSG is a difficult process and a significant proportion produced has commonly to be discarded because it is of insufficient quality. This might explain partly the high cost of branded antimonials (US$150–200 per patient for a full drug course), and gives food for thought as to the origin of toxic generic batches. Generic SSG was advocated as a solution to the drug access problem in developing countries, because of its better affordability (US$13–16 per patient per course). In that regard, recent studies comparing the generic SSG (SAG) from Albert David Ltd and the branded SSG (Pentostam® from Glaxo-Wellcome, London, UK) performed in Kenya, Sudan, and

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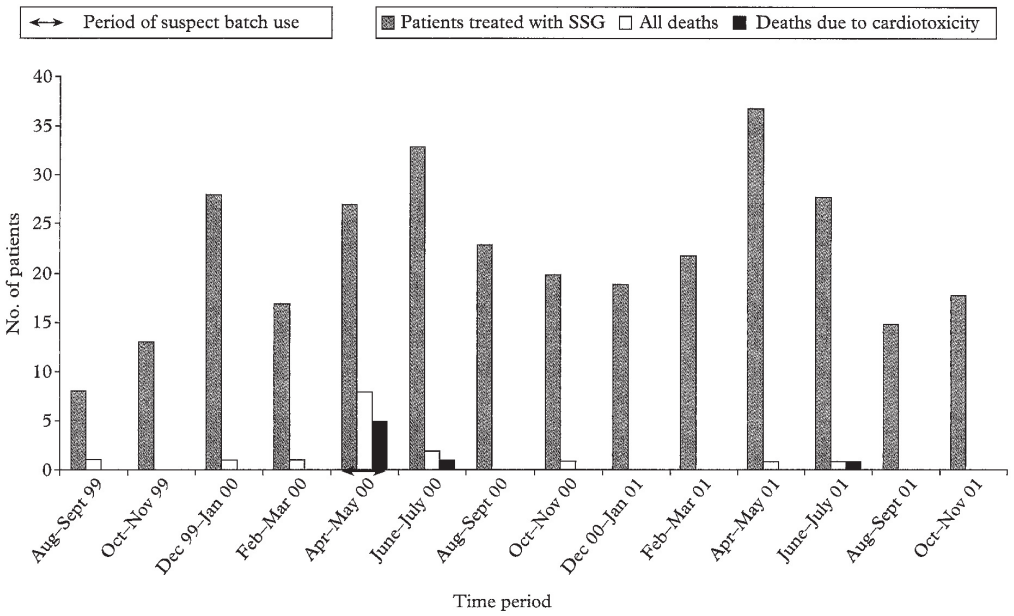

Figure. Number of patients, total deaths, and deaths due to cardiotoxicity amongst visceral leishmaniasis patients treated with generic sodium stibogluconate at the B. P. Koirala Institute of Health Sciences, Dharan, Nepal from August 1999 to December 2001.

Ethiopia showed equivalent efficacy and toxicity (Veeken *et al.*, 2000; Moore *et al.*, 2001; Ritmeijer *et al.*, 2001). The use and distribution of cheaper generic antimonials should thus be strongly promoted but this should be done with appropriate assurance of safety and efficacy. We believe that generic SSG should be widely used in VL-endemic countries but that rigorous quality controls should be performed on every batch to prevent outbreaks of fatal complications as reported here. For example, independent quality control of every single batch of SSG from Albert David Ltd is currently being implemented by the International Dispensary Association, Amsterdam, The Netherlands before distribution to East African countries. Also, the use of lower total doses of SSG by using combination therapies (e.g. SSG with paromomycin) might efficiently decrease its dose-dependent cardiotoxicity.

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CHAPTER 6:

GENERAL CONCLUSIONS

6. General conclusions

The thesis summarizes the clinical and epidemiological research on VL we conducted at the B.P. Koirala Institute of Health Sciences, Dharan, Nepal over a period of 6 years. This university hospital located in the terai, lowlands, has established itself as a specialized VL centre, attracting VL patients from a wide area. The centre has also taken a lead to conduct need based research to generate evidence for the better management and control of kala-azar.

6.1 The burden of visceral leishmaniasis in Nepal

We have documented, from observations over the preceding 5 years, that the VL endemic in Nepal shows no signs of decline in spite of an ongoing governmental control program for more than 10 years. In addition to VL being well established in the already known endemic areas we provide evidence that signals a possible further extension that should be urgently examined. We have also described how economically catastrophic the disease can be to the affected households.

The goal of the national health policy, to reduce VL incidence to less than 1 in 100,000 person-years by the year 2018 would look futile in the present perspective unless remedial steps are taken. The reasons for failure to bring the disease under control could be linked to multiple factors. In addition to the weaknesses in the implementation of the present control measures, other factors such as patient behavior, changing ecology and the regional political context need to be taken into consideration.

The current strategy of the VL control program has mainly focused on vector control using indoor residual insecticide spraying (IRS) along with early case detection and appropriate treatment. Intensive campaigns, using “blanket spraying” of all houses in the region, decreased the incidence of VL in India and Bangladesh to undetectable levels in the 1960’s (Bora 1999). However, these results have not been replicable

during routine activities, where reliable surveillance data and information on effectiveness of insecticides is crucial.

In Nepal, IRS activity is limited to high VL transmission foci identified from routine surveillance data. However, complete and adequate surveillance data for VL in the subcontinent has been lacking mainly due to underreporting and under-diagnosis. Desjeux (1991) found a 1:5 ratio of declared to undeclared VL cases in community surveys in India. In Nepal only those patients attending the public health facility are recorded in the MOH surveillance network, the cases attending the private sectors and other institutions do not get included (Bista 1998). The fact that we observed several cases in our tertiary care centre from non-endemic regions hitherto was not reflected in the concurrent surveillance data of the MOH. This indicates the limitations of the present surveillance system for VL, which does not extend beyond the known endemic districts.

Information on the effectiveness of the insecticides used becomes particularly important in view of some reports from Bihar, India, of resistance of the sandfly to DDT (Mukhopadhyay et al. 1990; Singh et al. 2001).

An alternative to residual household spraying with insecticides for the control of sandfly could be the use of insecticide treated bednets (ITN). Over the last two decades ITN have been shown to be one of the most effective methods of reducing man-vector contact and intra- and peridomiciliary transmission of malaria (Lengeler 1998). In a landmark study from The Gambia, use of ITN reduced childhood mortality by 60% (Alonso et al. 1991). However, the benefits conferred by ITN depend greatly on the biting habits of the vector along with human behavior. In the hilly and forested area of central southern Vietnam, where the main vector is exophagic and exophilic, ITN had limited impact in the prevention of malaria among the forest workers (Erhart et al. 2004).

The insecticides used in ITN (synthetic pyrethenoids) combine the properties of low to moderate mammalian toxicity, low volatility and high insecticidal activity. The ITNs in addition to acting as 'baited traps' also have important repellent and deterrent effects and also overcome some of the disadvantages of IRS. They require

less insecticide and as the households exert control over its application there is less dependence on a top-down planned control programme. The periodic re-impregnation of insecticides in ITN has now been partially overcome by the long-lasting-insecticide-treated-nets (LLIN) in which the insecticide is combined with the material during the manufacturing process.

The endophagic behavior of *Phlebotomus argentipes*, the sandfly vector in the Indian subcontinent, along with its night biting habits provides a rationale that ITN would potentially be protective in this region. There is experimental evidence of ITNs reducing the sandfly biting rate by 64 to 100% (Murray et al. 2005) and their effect on VL is presently being evaluated in the Indian subcontinent. Although it may not be justified to advocate its use yet in the routine VL control programmes at present, LLIN has the potential to be an extremely valuable tool for sandfly control.

The lack of regular supply of anti-VL drugs has been a major obstacle for access to drugs within the region. In Bangladesh, SSG shortages both in the government supply system and in the private sector were common during the last few years (Ahluwalia et al. 2003). The VL control programme in Nepal is a priority -1 category programme of the MOH, and they have been able to supply and distribute free anti-VL drugs quite regularly to the kala-azar endemic districts. But it has not been effective in making them available at the primary level health care facilities. We observed that majority of the patients with past history of VL received their treatment at or above a district level hospital. For most VL patients coming from remote rural areas and surviving on daily wages, the loss of earnings along with the added cost of transport and hospitalization makes access to these hospitals very difficult. Moreover, with little knowledge of the disease (Koirala et al. 1998) they prefer to visit the traditional faith healers or local unqualified private practitioners. We showed that this not only delays specific VL treatment but also lead to catastrophic expenses. In addition to the difficulties of access to the drugs, there is at present no proper system for monitoring the completeness of the therapy.

The contiguity of the VL endemic regions in the state of Bihar, India and Nepal along with socio-cultural similarity has facilitated large scale migration of populations,

which is an important risk factor for spread of the disease within the region (Desjeux 2001). Moreover, the ongoing violent conflict and increasing poverty has further accelerated migration including to the urban areas. We have observed increasing number of cases from the urban areas, mostly confined to newly established and unorganized settlements.

An important sequel following kala-azar, which may be possibly related to inadequate treatment, is post kala-azar dermal leishmaniasis (PKDL). Though patients with this condition have been recognized as an important reservoir for VL, it remains a neglected part of the control program. PKDL is a well recognized entity in Nepal (Garg et al. 2001) but there is no provision for the diagnosis or treatment within the present control program.

It would look irrational to single out one disease from the "burden" of many tropical diseases as they are often interrelated in their impact on the population's health. However, the implications of VL on those affected communities can be devastating. The model with the cycle of illness leading to poverty which then subsequently blocks economic development in addition to predisposing the affected households to further disease can be clearly demonstrated with this disease.

Though there has been a strong political commitment from the MOH, the lack of adequate resources has been a major deterrent to implement or upgrade the existing control program. The recently launched "Elimination of kala-azar from endemic countries from South-East Asia region" (TDR 2005) brings an opportunity to overcome these present constraints and facilitate in upgrading the existing control program. It also presents a platform to strengthen regional collaborations for VL control within the subcontinent.

6.2 Novel tests for early diagnosis of VL

The challenge for VL diagnosis has been to find a test that can be easily applied at the level of a peripheral health facility. This led to development of serological tests,

including DAT and rK39 strip test, over the last 2 decades but these failed to be adopted due to lack of consensus on their performance. The results varied depending on the phase of development, region and the manufacturer.

An ideal test should be sensitive, specific, reproducible, feasible and cheap. We showed both the DAT and the rK39 strip test have sufficiently high sensitivity and specificity and can be recommended as a diagnostic test in the south-east Asia region. We could overcome the bias associated with the use of an imperfect gold standard, which may have been one of the factors for the discrepancies in the assessment of these tests earlier on, by applying LCA, a mathematical modeling technique. In addition to the higher cost of DAT compared with the rK 39 strip test, its main limitations has been the unavailability of a commercial kit (Guerin et al. 2002) and the relative sophistication which precludes its use in the peripheral health care centres. This is not the case with the rK39 strip test. Subsequently, we also showed the rK39 strip test had good sensitivity, specificity and reproducibility even when evaluated at a peripheral level hospital, the site where we expect most VL patients to attend. The evidence from our research was instrumental in recommending the rK39 strip test as a diagnostic test in the control programme in Nepal in 2002.

An antigen based test like KAtex could become a major break through for VL diagnosis as it overcomes the limitations of the serological tests. However, we found that it has for the moment a low sensitivity although, interestingly, a positive correlation with parasite load was seen. The excellent sensitivity of KAtex seen in VL-HIV co-infected patients (Riera et al. 2004) may be related to a higher *Leishmania* parasitemia in these patients. With improved sensitivity and reproducibility this test could be very useful as it would obviate the need for repeating the parasitological examination to assess cure after completion of therapy.

The potential impact of the availability of an accurate diagnostic test that can be easily applied at a peripheral level hospital cannot be overemphasized. The benefits extend from decreasing the morbidity and mortality related to delay in diagnosis (Collin et al. 2004) to reducing the cost related to the disease. We observed how the

majority of the costs related to VL treatment occurred even before the disease was diagnosed. Moreover, with humans being the only known reservoir in anthroponotic VL, early diagnosis facilitating effective treatment is considered a significant measure to control disease transmission (Guerin et al. 2002).

6.3. Efficacy of the current VL treatment in Nepal

We documented the cure rates with SSG, the first line therapy, in Nepal. They were significantly lower in cases coming from areas close to the Bihar high resistance focus when compared to the other areas. Similarly, the mortality with treatment was also much higher in cases from this region.

The failure of therapy could be related to use of inadequate or incomplete treatment schedules, development of acquired resistance of the parasite to SSG or decreased immune status of the patient. In our study the adequacy of the therapy was ensured and the incidence of *Leishmania*/HIV co-infection was negligible, so emergence of acquired resistance is suspected, which can only be confirmed by performing drug monitoring tests.

In the adjoining state of Bihar, a steady decrease of SSG cure rates, even with the maximum recommended doses, has been reported since the early 1990's (Sundar 2001) and this has been linked to development of resistance of the L*eishmania* parasite to SSG (Lira et al. 1999) which is reported to be widespread in Bihar (Thakur et al. 2004).

Evidence of increasing failure rates of SSG in some areas in Nepal may suggest that the situation in Nepal may follow the same path of drug failure as that seen in the state of Bihar, India unless immediate remedial steps are taken. The major factors which may have directly or indirectly contributed to SSG treatment failure in Bihar include lack of sustained and effective control programmes, inadequate and/or incomplete therapy and emergence of resistance strains of *Leishmania* parasite.

The control measures implemented with the start of the epidemics in Bihar (1977 and 1991-92) were not only discontinued after only 3 years (Thakur 2005) but it was

also observed that 57% of the houses had not in fact been sprayed with DDT between 1992 to 1994 (Thakur 2000). The unabated intense transmission leading to the presence of a large biomass of parasites has contributed to the steady increase in primary resistance to SSG observed in Bihar (Bryceson 2001) . The areas showing lower cure rates in Nepal, in addition to being closer to the Bihar high resistance zone, also have more intense transmission as they have experienced the epidemic for a longer duration compared to the other areas.

The shortcomings in the implementation of the control program in Bihar also include delivery of drugs to patients as documented in a study, which reported that only a quarter of the patients received adequate SSG treatment, most of the patients had been treated by unqualified practitioners and almost all the patients used their own resources to buy the drug (Sundar et al.1994). The patients who failed therapy in this study were found to receive sub-therapeutic or incomplete doses, which is considered an important factor for the development of secondary resistance to SSG (Bryceson 2001). It would thus appear that having an effective control programme is crucial not only for reducing treatment failure but also for prevention of development of drug resistance.

The availability of generic brands of SSG has had a significant effect on bringing down the cost of treatment without compromising the safety or efficacy of therapy when implemented with drugs from bonafide manufacturers. We showed the need for establishing rigorous quality control measures of generic drugs to prevent serious toxicity and deaths related to substandard and toxic products.

The present evidence of low cure rates of SSG implicates the need for reviewing the drug policy for VL treatment in Nepal. The presently available options to replace SSG include amphotericin B or miltefosine, both of which have shown excellent cure rates in recent studies conducted in the Indian subcontinent (Jha et al. 1999; Rijal S et al. 2005; Thakur et al. 1999). In addition to its toxicity, the need for prolonged hospitalization for amphotericin B could be a major limitation in the face of limited hospital beds. The oral drug, miltefosine, overcomes this disadvantage but needs to

be used with caution in women of childbearing age because of its teratogenic potential. Strengthening of the drug distribution and monitoring would be paramount before this drug could be safely brought into the existing control programme.

In summary we have documented that the VL epidemic in Nepal continues unabated, provided evidence for the validity and feasibility of using a rapid test, rK39 strip test, at peripheral level hospital and showed that the present drug regimen is no longer effective. Our research work has also brought into focus some important issues which would need to be pursued further. Epidemiological and entomological studies need to be conducted urgently to confirm the presence of new foci and to measure the intensity of transmission. There is also an immediate need to monitor *Leishmania*/HIV co-infections. Establishment of regular monitoring of drug resistance as described by Croft (Croft 2001) would be vital to guide a rational drug policy.

What are the implications of our observations from Nepal for the other anthroponotic VL areas in the Indian subcontinent and east Africa? In similar socio-economic environments, the devastating economic consequences of the VL disease will be similar; as was already observed in Bangladesh (Ahluwalia et al. 2003). It is also expected that the performance of serological tests (rK39 strip test and DAT) in these regions would be as good as observed in Nepal with regard to sensitivity, but the specificity may be somewhat lower if there is an increased prevalence of VL antibody in the general community in regions which have experienced prolonged and intense transmission of the disease e.g. Bihar, India. In this scenario, though the negative predictive value may remain good, the positive predictive value of these antibody detection based tests being lower would necessitate the use of more specific tests as an antigen detection test or else the parasitological confirmation in a second step. In fact, the diagnostic policy in Bihar, India presently combines a serological test along with a specific biological criteria *e. g.* lecopenia. As efficacy of SSG varies in different regions and sometimes even within the same country (Sundar et al. 2000) extrapolation of our results is not appropriate to guide drug

policy elsewhere. It is absolutely necessary for the national control programmes to set up effective monitoring of efficacy of the VL drugs.

In addition to the emergence of drug resistance, increase in *Leishmania* /HIV co-infections would pose serious threats for the control of VL in future. This co-infection reinforces the effects on the immune system making it difficult to make a serological diagnosis and also to achieve cure. The spread of the AIDS pandemic from the urban to rural areas along with simultaneous spread of VL from rural to urban regions augments the risk for this co-infection.

Though there have been some exiting advances in recent years for controlling this neglected disease with the availability of serological tests and new drugs like miltefosine much more needs to be done. More affordable field tools (including antigen detection tests) for rapid diagnosis customized for specific geographical regions and tests for predicting prognosis and early detection of relapses are required. Development of molecular probes for detection of drug resistant strains would be a useful tool in the light of emergence of drug resistance strains. Moreover, as in malaria, adopting combination therapy for VL seems the most logical way to preserve the current and future drugs from becoming resistant.

Finally, we have emphasized the need for strengthening the existing health system including the surveillance network as the successful delivery of any control strategy is ultimately dependent on the performance of the health system.

6.4 References

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